Cationic polypeptides for gene delivery to eukaryotic cells

Lucas, Paul

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Cationic Polypeptides for Gene Delivery to Eukaryotic Cells.

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
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Summary.

Among the potential non-viral vectors for human gene therapy are polypeptide-DNA systems. In this study the factors affecting gene transfer by this method have been evaluated. Complexes of plasmid DNA with poly-L-lysine, streptavidin-poly-L-lysine, alanine-lysine graft co-polymers and histone H1 were prepared by direct mixing and examined by spectrofluorimetry and agarose gel electrophoresis. Addition of polypeptides to DNA yielded complexes that progressively excluded the fluorescent probe ethidium bromide and exhibited marked reductions in electrophoretic mobility. For each polymer the cation-to-DNA phosphate ratio required to produce DNA condensation was determined. For poly-L-lysine homopolymers and histone H1, condensation was induced at peptide cation-to-DNA phosphate ratios close to electrostatic equivalence.

Complexes formed between polypeptides and plasmid DNA carrying the *lacZ* reporter gene (pRSVlacZ) were used to transfect B16 melanoma cells *in vitro*. Polylysine(127), (214), (859) and histone H1 were effective intra-cellular gene delivery agents, while polylysine(13) and alanine-lysine graft co-polymers were ineffective. A critical dependence of gene transfer efficiency on the polycation-to-DNA ratio was established. Zeta potential measurements confirmed gene transfer was charge-mediated with transfecting polypeptide-DNA complexes requiring an excess of positive charge. Consistent with the cellular uptake of polypeptide-DNA complexes through endocytosis, the expression of the *lacZ* gene included in the transported DNA was shown to be dependent on the presence of the lysosomotropic agent chloroquine. Stability experiments showed polypeptide complexed DNA was protected from degradation by serum. Other factors which effect gene delivery and expression include complex morphology, carrier molecular weight, DNA dose, and dosing schedule.

As an alternative strategy, the feasibility of delivering DNA to B16 melanoma cells by means of receptor-mediated endocytosis was tested. Molecular conjugate vectors which employed concanavalin A for targeting produced a modest but significant increase in *lacZ* expression compared to a control vector. However, a more specific system incorporating N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH, a ligand at the MC 1 receptor expressed on melanoma cells and melanocytes, failed to produce increases in delivery efficiency.

For Mum and Dad.

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Abbreviations.

Å	Angstrom(s)
AMPS	ammonium persulphate
BSA	bovine serum albumin
bp	base pairs
Da	daltons
dAMP	deoxyadenosine-monophosphate
dCMP	deoxycytidine-monophosphate
dGMP	deoxyguanosine-monophosphate
dTMP	deoxythymidine-monophosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DOTMA	*N*-[1-(2,3-Dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride
DPn	Degree of polymerisation
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	ethylene diamine tetra-acetic acid
FCS	fetal calf serum
FDG	fluorescein di-β-D-galactoside
FITC	fluoresceinisothiocyanate
g	gramme(s)
HEPES	*N*-(2-hydroxyethyl)piperazine-N-2'-ethane sulphonic acid
Hz	Hertz
k	kilo
k_{da}	dissociation constant of radiotracer
k_{db}	dissociation constant of competitor ligand
LB	Luria broth
μ	micro
M	mole(s) per litre
M_n	number average molecular weight
M_w	weight average molecular weight
MBq	mega-bequerel(s)
mg	milligram(s)
mRNA	messenger RNA
MOPS	3-[N-Morphilino]propane-sulphonic acid
nm	nanometre(s)
OD	optical density
ONPG	o-nitrophenyl-β-D-galactoside
PAGE	polyacrylamide gel electrophoresis
PE	phosphatidylethanolamine
pI	iso-electric point
pLL	poly-L-lysine
RNA	ribonucleic acid
rpm	revolutions per minute
S	siemens
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
TE	Tris-EDTA buffer
TEMED	N,N,N,'N'-tetramethylethylene diamine
Tris	tris(hydroxymethyl)aminomethane
TFA	trifluoroacetic acid
UV	ultraviolet
V	volt(s)
$^{v}/_{v}$	volume by volume
$^{w}/_{v}$	weight by volume
X-gal	5-bromo-4-chloro-3-Indolyl-β-D-galactopyranoside

Chapter 1

Introduction.

It is now widely accepted that the basis of many diseases is the development or inheritance of abnormalities in the cellular genetic code. This understanding, brought about by recent advances made in the sciences of molecular biology and human genetics, is likely to revolutionise the approach to treating disease. Although many advances have been made in medicine the vast majority of therapies, with the exception of surgery and anti-microbial chemotherapy, have been aimed at treating the chemical or biochemical consequences of a disease (Friedmann, 1993). However, gene therapy is based on the correction of a disease phenotype at the level of the responsible defect. Gene therapies were originally proposed for monogeneic dysfunctions where the clinical manifestations of a disease could be treated simply by replacing the defective gene. However, more recently genes have been inserted into eukaryotic cells in order to express pharmacologically active proteins *in situ*.

1.1. Single gene defects as targets for gene therapy.

The initial target diseases for treatment by gene therapy were those which resulted from deficiencies or mutations in a single gene. Perhaps the most extensively studied of these is cystic fibrosis (Schreier, 1994). This disease of the exocrine glands results from mutations in the cystic fibrosis transmembrane conductance regulator gene (Riordan *et al*, 1989) that encodes a cyclic AMP-regulated chloride ion channel (Welsh *et al*, 1990). The CFTR gene has been transferred *in vivo* to the airways of animal models using a number of delivery systems with the restoration of cAMP mediated Cl^- efflux (Hyde *et al*, 1993; Alton *et al*, 1993). Several of the subsequent clinical trials in cystic fibrosis patients have also reported the successful

delivery and expression of a functional gene product in the nasal epithelia of subjects so indicating the clinical potential of gene therapy for this disease (Zabner *et al*, 1994, Caplen *et al*, 1995). Other single gene defect diseases proposed as targets for treatment include α_1-anti-trypsin deficiency (Kay *et al*, 1992), Gaucher's disease (Ohashi *et al*, 1992) and Lesch-Nyhan syndrome (Mulligan and Berg, 1981).

1.2. Gene therapy for neoplastic disease.

Gene therapy is one of a number of novel biological approaches to the treatment of neoplastic disease that are likely to supplement the more traditional therapies of surgery, radiotherapy and chemotherapy. Indeed, the first approved gene transfer experiment was conducted in patients with advanced malignant melanoma although in this study the aim was to track tumour-infiltrating lymphocytes expressing a marker gene (Rosenberg *et al*, 1989). In similar corrective strategies to those discussed in section 1.1. experiments have been conducted where mutant genes have been replaced with a single functional copy. Using this technique the *in vivo* proliferation of a number of human tumour cell lines was prevented following transfection with a wild type p53 gene which encoded a phosphoprotein with tumour suppressor activity (Mercer *et al*, 1991). However, as the tumourigenicity of cells is unlikely to result from a single mutation then it may be necessary to target two or more genetic defects simultaneously (Vile and Russell, 1994). These corrective strategies require highly efficient gene transfer as each individual cell must be targeted.

1.2.1. Genetic immunomodulation.

A novel adaptation of gene therapy applied to the treatment of cancer has been the vaccination of subjects with tumour cells modified *ex vivo* with genes encoding molecules of the major histocompatability complex. The use of cancer vaccines has

been reviewed extensively by Dalgleish, (1994). The principle of this strategy is that the presence of expressed molecules on the surface of transfected cells enhances their immunogenicity and results in recruitment of immune cells to the tumour mass and the killing of cancer cells. This approach has recently been extended to the direct modification of tumour cells *in situ* by *in vivo* transfer of allogenic MHC genes (Plautz *et al*, 1993).

There has also been considerable interest in inducing tumours to constitutively secrete cytokines such as alpha-interferon and Interleukin 2 in order to increase the tumour killing properties of the immune system. Indeed, there have been several reports indicating that the insertion of the IL2 cDNA in murine tumours results in the generation of an anti-tumour response (Fearon *et al*, 1990; Gansbacher *et al*, 1991). Conceptually, this technique gives an anti-tumour response by generating cytokine concentrations in the tumour *local* which could otherwise only be achieved by the infusion of high doses of recombinant protein. The latter strategy is associated with severe systemic side-effects (Foa *et al*, 1992).

1.2.2. Gene directed enzyme pro-drug therapy.

One of the most promising applications of gene therapy to the treatment of cancer is the selective targeting of drugs to tumours using pro-drug therapy (for a review see Deonarain *et al*, 1995). In this technique a foreign gene encoding an enzyme capable of converting an inactive pro-drug into a cytotoxic compound is introduced into tumour cells. Several strategies for achieving selective delivery, including targeting with immunoliposomes or viruses with predetermined tissue specificity, were proposed in a review by Gutierrez *et al*, (1992). However, the method of selection preferred by these workers was to restrict the expression of the gene to target cells possessing the correct transcriptional control elements (Harris *et al*, 1994). Using

vectors containing the gene for cytosine deaminase under the control of the *ERBB2* promoter sequence selective conversion of 5-fluorocytosine to 5-fluorouracil was seen in *ERBB2* expression positive pancreatic and breast tumour cell lines; significant cell death was observed in *ERBB2*(+) cells whilst *ERBB2*(-) cells remained viable.

1.2.3. DNA vaccination.

Gene therapy has also been proposed as a strategy for the generation of protective immunity against viral infections. In a recent advance the direct injection of plasmid DNA encoding the influenza A nucleoprotein into muscle resulted in both gene expression and the generation of antigen-specific cytotoxic T-lymphocytes (Wolff *et al*, 1990). The resulting cell mediated immunity, which appeared not to be strain specific, protected the animal model against a lethal viral challenge (Ulmer *et al*, 1993). Therapeutic gene vaccines have also been developed as one of a number of gene therapies for HIV infection (for a review see Yu *et al*, 1994). A trial involving the retroviral delivery of the gene encoding HIV-1 gp160 outer coat protein has now advanced to the clinical stage.

1.3. Gene transfer.

Gene transfer to eukaryotic cells may be accomplished either *ex vivo* or *in vivo*. In the first situation, target cells are removed from the patient, isolated, and expanded in culture before being genetically modified. The explanted cells, which now express the desired gene, are then returned to the patient. This technique has generally been used in the treatment of haematological disorders (e.g. adenine deaminase deficiency), where the target cells are directly accessible, or for the modification of immune cells prior to tumour vaccination. Under these conditions it is possible to

use gene transfer strategies such as electroporation and calcium-phosphate-DNA co-precipitation (Graham and Van der Eb, 1978) which are unacceptable *in vivo*. However, this technique requires cell lines from each patient to be established in the laboratory which delays treatment and makes the method difficult to apply in common clinical practice (Plautz *et al*, 1993). In contrast, *in vivo* gene transfer involves the delivery of the therapeutic gene directly to the target cells at the level of the affected tissues or organs. This approach could expand the practical applications for gene therapy and would in essence be similar to current pharmaceutical treatments. However, whilst this strategy is conceptually more convenient it places significant additional requirements on the gene delivery system.

1.3.1. Properties of the ideal delivery system.

For *in vivo* gene therapy to succeed as a strategy for treating disease it is widely recognised that the therapeutic gene must be reproducibly expressed in the target cells at a level sufficient to achieve a therapeutic effect (Dillon, 1993; Mitani and Caskey, 1993). Therefore, the delivered gene must be efficiently presented at the site of action (i.e. inside the nucleus) which requires transport across multiple sub-cellular barriers. These include obtaining access to the target tissue, transit through the plasma membrane, transport to the nucleus via the cytoplasm and nuclear entry (Michael and Curiel, 1994). It is also important to consider the design of the expression module (therapeutic gene+promoter/enhancer elements) which is an integral part of the delivery system since it regulates the transcription of the therapeutic gene. The choice of promoter has been shown to influence significantly the level of gene product expression *in vivo* (Davis *et al*, 1993). In addition, several other intrinsic characteristics are required by the delivery system to accomplish *in vivo* gene transfer successfully. The requirements for an ideal gene transfer system are as follows (Michael and Curiel, 1994; Rolland *et al*, 1994):

(i) the delivery of therapeutic genes to the target cell must be efficient and reproducible.

(ii) the vector system must be stable *in vivo* and either by means of selective delivery or expression, the system must exhibit specificity for the target site.

(iii) the vector system incorporating the therapeutic gene must be internalised by the target cell and gain access to the cytoplasm.

(iv) the vector system must allow transfer of DNA from the cell cytoplasm to the nucleus and express therapeutic gene.

(v) the vector system should be non-toxic and biodegradable (synthetic vectors).

(vi) the vector system must lack immunogenicity and must not be recognised by the reticuloendothelial system.

(vii) the vector system, as a pharmaceutical formulation, needs to be physically and chemically stable.

1.3.2. Current technologies for gene delivery.

A list of the major approaches to delivering genes, with selected references, is shown in Table 1.1. The techniques proposed currently for gene transfer are based on either viral transduction or physical transfection (Hodgson, 1995). In transduction, the therapeutic gene is introduced into the target cell genome following viral infection. Alternatively, transfection uses either physical of particulate methods to transfer the foreign gene into the cell nucleus.

Table 1.1. Gene transfer techniques. Significant reviews and early publications considering these techniques and their applications are cited.

Strategy	Method	Reference
Viral		
	Retroviruses	Shimotohno and Temin (1981)
		Eglitis and French Anderson (1988)
	Adenoviruses	Berkner (1988); Ali *et al*, (1994)
	Adeno-associated virus	Flotte and Carter (1995)
	Herpes simplex virus	Geller and Breakfield (1990)
	Vaccinia virus	Sutter and Moss (1992)
Non Viral		
Direct	Electroporation	Andreason and Evans (1988)
	Micro-injection	Graessmann and Graessmann (1986)
	Micro-particle acceleration	Johnston (1990)
Particulate	Plasmid DNA	Wolff *et al*, (1990); Ulmer *et al*, (1993)
	Calcium phosphate precipitation	Graham and Van der Eb (1975)
	Liposomes	Felgner *et al*, (1987); Gao and Huang (1991)
	Cationic polypeptides	Smull and Ludwig (1962); Farber *et al*, (1975)
	Poly amido acid cascade polymers	Haensler and Szoka (1993)

1.4. Viral Vectors.

Viral methods for gene transfer have their origins in the discovery that a series of 'tumour viruses' produced their effects by integrating their genetic material into the genome of the infected cell (Westphal *et al*, 1968). Therefore, viral gene transfer systems capitalise on the efficient internalisation pathway of the progenitor virus for gene delivery. Since the development of retroviral vectors (Shimotohno and Temin, 1981), delivery systems based on adenovirus (Berkner, 1990), adeno-associated virus (Flotte and Carter, 1995), herpes simplex virus (Geller and Breakfield, 1990) and vaccinia virus (Sutter and Moss, 1992) have been used for gene transfer. Although not part of this study retroviral and adenoviral vectors are considered as they are the most commonly used systems for *in vivo* gene transfer.

1.4.1. Retroviruses.

The majority of approved clinical trials of gene therapy have involved the use of retroviral vectors for gene transduction (Davies, 1992). In this context the vectors are retroviruses (RNA viruses) in which the genome has been altered so that viral proteins are not made in cells infected with the vector. Using this method it is possible to achieve a high efficiency of gene transfer with approaching 100% of dividing cells being transduced in culture (Mitani and Caskey, 1993). These vectors have been used in the experimental treatment of lysosomal storage diseases (Ohashi *et al*, 1992) and in selective cancer therapies (Huber *et al*, 1991). However, the use of retroviral vectors is associated with a number of problems that are inherent in the biological properties and life-cycle of the virus.

Primarily, a round of cell division is required shortly after infection for the integration of pro-viral DNA into the cell genome (Miller *et al*, 1990). This serves to effectively reduce the transfer efficiency of the technique especially for the transduction of cells *in situ* where the rate of cell division is significantly less than in

culture. Additionally, the very nature of this integration is of concern for although the process confers permanency of expression on transduced cells it has been linked to the insertional activation of oncogenes (Donahue *et al*, 1992). In the latter study, three out of the ten monkeys which received retrovirally transduced bone marrow progenitor cells developed a T-cell lymphoma. However, it is possible this was an isolated case, which resulted from infection of subjects with a retroviral supernatant containing a high titre of replication competent retroviruses (Anderson, 1993). It is, therefore, necessary that vector-producing cell lines must undergo extensive testing for the presence of replication competent retroviruses.

The size of the foreign gene which can be accommodated into retroviral vectors is limited as RNA is packaged within the virion. The amount of exogenous DNA which recombinant retroviral vectors can package is limited to 6-7 kbp (Eglitis and Anderson, 1988). Additionally, these enveloped viruses undergo complement mediated inactivation in the presence of serum. As such it is likely that even if retroviral vectors can be highly purified their use will remain confined to the *ex vivo* transfer of genes in culture systems where their high efficiency can be utilised.

1.4.2. Adenovirus.

Adenoviruses are a group of non-enveloped DNA-containing viruses with spiked icosahedral morphology. Though a large number of adenoviral serotypes exist the adenoviral vectors for gene transfer are generally constructed using the non-oncogenic types 2 and 5 (Ali *et al*, 1994). Systems of this type have been used successfully for *in vivo* gene transfer to airway epithelial cells (Rosenfeld *et al*, 1992) and skeletal muscle (Ragot *et al*, 1993). The construction of adenoviral vectors allows the accommodation of up to 7.5 kbp of foreign DNA although manipulation of the viral genome has shown this can be increased to 9.3 kbp (Armentano *et al*, 1994).

Adenoviral vectors are radically different from retroviruses as they are capable of infecting both quiescent and terminally differentiated cells such as hepatocytes and neurones (Jaffe *et al*, 1992; La Gal La Salle *et al*, 1993). However as the adenoviral genome does not generally undergo integration following infection, foreign genes are transiently expressed. Given this situation the repeated administration of adenoviral vectors will be required in chronic therapies. Importantly, multiple challenges with adenovirus particles have resulted in the development of anti-adenoviral antibodies in animals (Kass-Eisler *et al*, 1994; Yei *et al*, 1994). Presumably it is this mechanism which reduces the efficiency of gene transfer and expression on repeated administration of adenovirus vectors (Smith *et al*, 1993; Yei *et al*, 1994).

Adenoviral vectors have also been associated with inflammatory immune responses. In experiments with primates this toxicity appeared to be dose related (Simon *et al*, 1993); the minimal inflammation that developed on administration of viral titres of 10^7 and 10^8 p.f.u. increased to more severe perivascular lymphocytic and histolytic infiltration at doses of 10^9 and 10^{10} p.f.u. Toxicity of this type has also been noted in clinical trials of adenoviral systems for the treatment of cystic fibrosis where effects have ranged from mild neutrophilia in the nasal cavity (Zabner *et al*, 1993) to more severe inflammation of the lower respiratory tract (Crystal *et al*, 1994). In one case inflammation subsequent to the aerosol delivery of adenovirus required the hospitalisation of a subject (FDA transcript, 1993).

1.5. Non-viral gene transfer.

As alternatives to viral vectors, non-viral delivery systems are being developed. These systems differ from viral delivery systems as they are generally based on plasmid DNA which is not 'infective' per se and, therefore, needs to be shuttled into the cell by some carrier system (Schreier, 1994); the physicochemical properties of plasmid DNA (high molecular weight, high charge density, sensitive to

hydrodynamic shear and nuclease digestion) generally reduces the efficiency with which it can enter into cells (Spooner *et al*, 1995). Conceptually, this approach has several advantages over the viral systems described in section 1.4 as the technical difficulties involved in the construction, manufacture and use of possibly pathogenic viral constructs is avoided (Felgner and Rhodes, 1991). Additionally, although permanency of expression is not generally achieved with non-viral systems this may be considered advantageous as it is possible to reverse a treatment that has resulted in undesirable side-effects (Wolff *et al*, 1990).

The non-viral gene transfer systems which are currently available are based on either the physical introduction of nucleic acids into the target cells or the use of synthetic particles. The first group of methodologies includes electroporation (Andreason *et al*, 1988), micro-injection (Graessmann and Graessmann, 1986) and microparticle bombardment (Johnston, 1990). Since these technologies may only be used for *ex vivo* genetic modification they are not discussed in detail. Those non-viral systems which are potentially useful for *in vivo* gene transfer have been based on naked DNA or synthetic particles such as liposomes or polypeptide carriers. Current research using polypeptide carriers is focused on encapsulating therapeutic genes into particle systems which are engineered to imitate viral mechanisms for cell entry and intra-cellular trafficking.

1.5.1. Plasmid DNA.

Plasmid DNA is prepared for gene transfer to mammalian cells by inclusion of an expression module containing the therapeutic gene and the necessary cis-acting elements including promoter and enhancer sequences as well as transcript processing signals (Vile and Russell, 1994). This technique has been used successfully to produce expression of dystrophin in muscle tissue (Cox *et al*, 1993) and to generate immunity (Wolff *et al*, 1990). DNA in this form is not expected to integrate into the

host cell chromosome. Rather, the DNA is intended to reside transiently as an episomal element during which time mRNA will be transcribed as a gene product (Ledley and Ledley, 1994). The advantages of this method are two-fold; first, large gene sequences can be introduced easily into the molecule and secondly plasmid DNA is itself weakly immunogenic and therefore can be administered repeatedly (Madaio *et al*, 1984). However, the efficiency of transfection with naked DNA is relatively low and it is likely that therapies will be limited to those, such as muscular disorders, where the site of administration is directly accessible.

1.5.2. Liposomal gene transfer.

The use of liposomes as vehicles for the delivery of polynucleotides began with neutral lipids but the use of this technique was limited by the low encapsulation efficiencies of the vectors (Felgner, 1990). Even with the use of large unilamellar vesicles only 30-50% of the input polynucleotide could be encapsulated (Szoka and Papahadjopoulos, 1978). However, it has been possible to circumvent these problems using polycationic liposomes comprised of positively charged lipids (Felgner *et al*, 1987; Behr *et al*, 1989; Gao and Huang, 1991). Since cationic vesicles interact avidly and spontaneously with negatively charged nucleic acids, the complete entrapment of DNA molecules is achieved at low liposome-to-nucleic acid ratios, thus obviating the difficulties related to the presence of empty liposomes in the transfection mixtures (Gershon *et al*, 1993).

Cationic lipid mediated transfection using small unilamellar vesicles formed with N-[1-(2,3,-dioleyloxyamino)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) and dioleoeylphosphatidyl-ethanolamine (DOPE) is now established as an efficient method for the delivery of both DNA and RNA to a wide variety of eukaryotic cell types (Felgner *et al*, 1987; Malone *et al*, 1989). This combination of lipids is now

available in the proprietary product Lipofectin®. Depending on the cell type, this methodology produced from 5- to >100 fold more gene expression than either the calcium phosphate or DEAE-dextran transfection techniques (Felgner *et al*, 1987). An alternative strategy is the use of liposomes prepared from DOPE and cationic derivatives of cholesterol (Gao and Huang, 1991).

Cationic liposome delivery of nucleic acids into the cell cytosol was initially postulated to occur by fusion with the plasma membrane (Felgner *et al*, 1987; Felgner, 1990). This hypothesis was proposed on the basis of *in vitro* experiments showing that vesicles comprised of DOTMA and DOPE, fuse with negatively charged lipid membranes and cell surfaces (Duzgunes *et al*, 1989). However, Legendre and Szoka, (1992) have shown that in CV-1 cells (monkey fibroblasts) treatment with the lysosomatropic agent chloroquine significantly increased cationic liposome-mediated gene transfer. These data suggest endocytosis was also a possible, and perhaps major, mechanism for the uptake of DOTMA/DOPE liposomes; similar results have also been described for cationic liposome systems with differing phospholipid compositions (Felgner *et al*, 1994). The release of systems from the endosomal compartment is believed to result from phosphatidylethanolamine-assisted membrane fusion (Felgner *et al*, 1994).

The utility of cationic liposomes has been further established by *in vivo* studies which have demonstrated cationic lipid mediated gene transfer to the airways (Alton *et al*, 1993; Logan *et al*, 1995), catheterised blood vessels (Nabel *et al*, 1992) and cells of the systemic circulation (Zhu *et al*, 1993). In addition, the *in vivo* toxicity of cationic lipid systems in several species appears to be minimal (San *et al*, 1993). However, despite liposomes representing significant progress toward the aim of acheiving effective *in vivo* gene delivery it is not yet clear whether these systems are sufficiently efficient to result in the necessary levels of gene expression for

therapeutic applications. Moreover, problems surrounding the pharmaceutical and serum stability of cationic-liposome-DNA complexes exist.

1.5.3. Synthetic or semi-synthetic cationic polymers.

The cationic polymers diethylaminoethyldextran and polybrene (a 5-10 kDa straight chain polymer containing quaternary amino groups) were originally used as a means of increasing the uptake of virus particles into cells possessing a net negative charge (Vogt, 1967; Toyoshima and Vogt, 1969). However, their polycationic nature has more recently been used to enhance the delivery of polyanionic polynucleotides to mammalian cells. The standard DEAE-dextran protocols have produced expression of the gene product in approximately 5-20 % of cells; however, the transfections are generally carried out on confluent cells to avoid the significant toxicity seen with this protocol (Danielsen *et al*, 1986). A less common procedure for the transformation of cells with DNA is the use of the synthetic polycation polybrene (Kawai and Nishizawa, 1984). Most protocols describing the use of DEAE-dextran or polybrene require the use of a mild hypertonic lysis step, using DMSO or NH_4Cl, which presumably increases membrane permeability at the level of the plasma membrane or the endocytic vesicles (Felgner, 1990); the latter procedures are unsuitable for *in vivo* gene transfer.

1.5.4. Cationic Polypeptides.

The use of cationic polypeptides to increase the uptake of functional polynucleotides followed from the work of Smull *et al*, (1961). In this study either histone (calf thymus) or protamine (200-1000 μg/ml) was pre-mixed with poliovirus RNA, under isotonic conditions at neutral pH, and then applied to HeLa cell monolayers. Both histone and protamine produced an increase in infectivity which was subsequently calculated to be approximately 100-fold greater than with the

native nucleic acid (Smull and Ludwig, 1962). Perhaps surprisingly, these workers also reported that some other cationic substances, including poly-L-lysine and spermine, appeared not to enhance RNA uptake. However, in a later and conflicting study, polyornithine, polylysine, and polyarginine appeared to facilitate the entry of nucleic acids into Chinese hamster lung cells in culture and uptake into a nuclear sub-cellular fraction was demonstrated (Farber *et al*, 1975). Higher molecular weight polymers were generally found to be the more effective than smaller polymers. A similar technique employing poly-L-ornithine (M_r = 100,000-200,000) was optimised for the transient and stable transformation of several cell lines (Bond and Wold, 1987). Efficiency of transfection was relatively low with 1 to 2% of the recipient cells taking up and expressing the selectable marker gene (thymidine kinase). However, in contrast to the vast majority of studies with cationic polypeptides and DEAE dextran the authors reported a close correlation between the transient and long term transfer efficiencies.

1.6. Studies on the action of cationic polypeptide carriers.

Early evidence from the studies of Smull and Ludwig, (1962) showed that the ability of basic polypeptides to increase RNA uptake by eukaryotic cells was only observed when nucleic acids were treated with the polypeptide before adsorption to cells. This suggested that positively charged proteins mediated their effects on gene transfer as a direct result of the interaction with the negatively charged nucleic acids (the theoretical basis of this interaction is examined in section 1.6.1.). Further support for this hypothesis is provided in several of the studies which have employed polycations in gene delivery. Common to these procedures was a critical dependence of gene transfer efficiency on the ratio of polypeptide to donor DNA. Furthermore, Felgner, (1990) observed that unlike the calcium phosphate co-precipitation technique the inclusion of carrier DNA in polypeptide-DNA formulations did not improve uptake. These observations infer that an excess of cationic polypeptide is

required to neutralise the negatively charged DNA polymer in a polypeptide-DNA complex so that the complex can interact with a cell surface that is negatively charged (Bell, 1978).

1.6.1. Interactions of polycations and nucleic acids.

The specific interaction of nucleic acids with synthetic polycationic polypeptides has been the subject of a number of studies motivated in a large part by the desire to understand the nature of the action of basic DNA binding proteins in the cell nucleus (Leng and Felsenfeld, 1966). The interaction of poly-L-lysine, protamine, arginine-rich histone IV and lysine-rich histone 1b with DNA was initially investigated using equilibrium dialysis (Akinrimisi *et al*, 1965). Data from this study indicated the importance of electrostatic factors in binding. Polylysine binding neutralises charges on DNA and produces dehydration as water molecules are excluded from the vicinity of the structure (Chang *et al*, 1973). This process can be prevented in solutions of high ionic strength (1.5 M NaCl; Carroll, 1972).

When cationic polypeptides are added to DNA it is apparent that the physicochemical properties of the system components are modified. In this regard it has been established that the melting temperature of DNA complexed with polylysine, polyornithine, polyarginine or lysine rich histones is significantly greater than that of naked DNA (Olins *et al*, 1967; Olins, 1969). Such findings indicate the stabilisation of DNA molecule strands on complexation. However, to effect this stabilisation a sequence of at least eight or more basic residues is required (Olins *et al*, 1968). The binding of DNA by these cationic polypeptides is also associated with structural phase transitions, with polypeptide-DNA complexes exhibiting markedly different behaviour to naked DNA on analysis by electrophoretic sedimentation (Tsuboi *et al*, 1966) and ultracentrifugation (Leng and Felsenfeld, 1966; Olins *et al*, 1967). Additionally, these studies suggest that at lysine/phosphate ratios less than

unity the binding of DNA and polylysine is co-operative. That is binding is non-random, such that when 0.5 equivalents of lysine are added per equivalent of nucleotide phosphate, half of the DNA and all the polylysine appear in a complexed state (Shapiro *et al*, 1969). From the analysis of systems formed between DNA and lysine rich histone (Olins and Olins, 1971) or polylysine (Olins and Olins, 1971; Laemmli, 1975) it is clear that the complexation event is associated with the formation of ordered structures with a compact configuration. In some cases the structures in these systems were toroidal (donuts). Circular dichroism studies published by Carroll, (1972) suggest the method used for the preparation of such systems may influence their physical characteristics.

1.7. Physicochemical characterisation of particulate systems.

Since the activity of particulate gene delivery systems is likely to be intrinsically linked to their physicochemical properties then the characterisation of these colloids is likely to become important in both the development and quality control of gene-pharmaceuticals. The colloid characteristics which are likely to be most influential are particle size and surface charge.

1.7.1. Electrical properties of colloids.

Surfaces of particles dispersed in a solvent become charged as a result of the adsorption of ions or ionisation of surface groups. These effects generate an electrical double layer around the particle in which the potential is highest near the surface and decays with the distance into the continuous phase (Fig. 1.1.). The charge on the surface of a particle is partially neutralised by co-ions in the stern layer. Each particle exposed to an electric field will move toward the appropriate pole and will carry with it an ionic environment which extends a small distance into the solvent. The potential at the surface of this layer (surface of hydrodynamic shear) is

termed the zeta potential (ζ) and is a practical measure of the potential on the colloid surface. The absolute potential (Stern potential) cannot be determined.

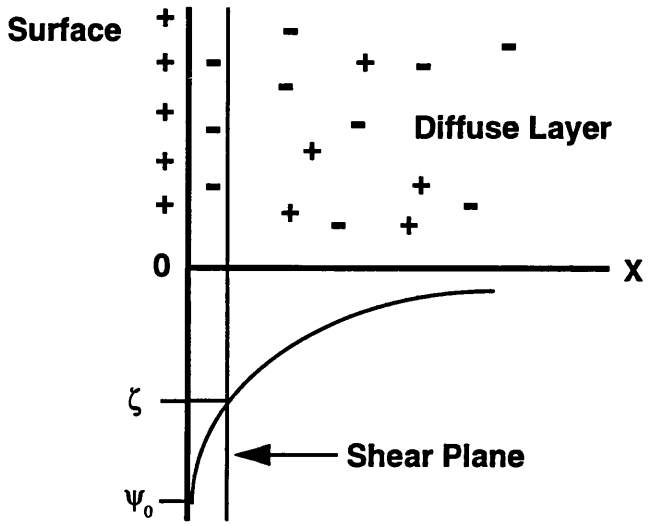


Figure 1.1. Representation of the conditions at a positive colloid surface, with a layer of adsorbed ions in the Stern plane. The potential at the particle surface is shown (ψ_0). As the particle moves the effective surface is defined as the surface of shear at which the zeta potential (ζ) is measured. Charge decays as a function of the distance (X) from the particle surface (adapted from Martin *et al,* 1970).

1.7.1.1. Measurement of surface charge.

The zeta potential on particles can be measured by a variety of techniques. However, the direct microscopic observation of particle movement under an applied electric field is tedious, error prone and is unsuitable for systems containing particles with sub-micron sizes (Washington, 1992). Therefore, this technique has largely been replaced by light scattering methods in which the velocity distribution of particles is measured from the Doppler shift of scattered light from moving particles. An extensive description of this technique is provided by Hunter, (1981). The zeta potential can then be determined using the Smoluchowski equation (Fig. 1.2.) which applies to large particles in weak electrolytes.

$$\zeta = \frac{\mu_c \eta}{\varepsilon_0 D}$$

Figure 1.2. Smoluchowski equation for large particles in weak electrolytes. ζ = zeta potential, μ_c the electrophoretic velocity, η the viscosity, D the dielectric constant of the suspending medium, and ε_0 the permittivity of free space.

1.7.2. Particle sizing techniques-general considerations.

A wide range of techniques have been applied to the study of particle size in pharmaceutical systems and these are reviewed extensively by Washington, (1992). However, the sub-micron size distribution of gene delivery systems makes photon correlation spectroscopy (PCS) and electron microscopy the most suitable methods for their characterisation. Scanning probe microscopy may also be a useful tool for the analysis of systems, though there is no published work available at present.

1.7.2.1. Photon correlation spectroscopy.

PCS is a light scattering technique that can be used in analysing particles from 10 nm up to a few micrometers in size. In this methodology, the fluctuations in scattered light intensity resulting from the Brownian diffusion of particles through a focused laser beam are used to calculate the diffusion coefficient of the particles. Small particles diffuse relatively rapidly which results in the fluctuations in scattering light being rapid. Conversely, the fluctuations in scattered light produced by large particles is relatively much slower. Data is collected by autocorrelation and a number of complex mathematical convolutions are then applied in order to determine the diffusion coefficient and calculate the equivalent diffusional spherical diameter of particles.

1.7.2.2. Electron microscopy.

Transmission electron microscopy is useful for the size analysis of sub micron particles but has a number of disadvantages (Washington, 1990). It is of limited use in the quantitative study of disperse systems, as obtaining numerical data on a total population of particles is both tedious and time consuming. However, the technique is more applicable in determining the gross morphological changes which take place within formulations. A further disadvantage of electron microscopy is the considerable sample preparation required. In the majority of published protocols, viral and liposomal particles are stained with heavy metals such as uranium or tungsten to increase sensitivity (Rolland, *et al*, 1994, March *et al*, 1995).

1.8. Targeting of gene expression.

The rationale behind the concept of gene targeting is to achieve a desired response from the expression of a gene product in selected cells without undesired actions at non-target sites. To this aim, two separate methodologies have been proposed for restricting the expression of genes to specific cell types. The first approach, transcriptional targeting, exploits observations that certain cells differentially and specifically express selected genes (e.g. gut epithelial cells/carcino-embryonic antigen; Schrewe *et al*, 1988). Expression of these genes is regulated by cell or tissue specific promoters. Therefore, by placing therapeutic genes under the control of such promoters it has been possible to restrict expression to a particular tissue (Vile and Hart, 1993). The specificity of gene expression can also be increased through the use of locus control regions (LCRs). These regulatory gene clusters direct the expression of transgenes in a copy dependent and integration site independent manner in different tissues. Cell specificity of LCRs was first demonstrated by Van Assendelft *et al*, (1989). In these experiments, expression of the β-globin gene under the control of a β-globin minilocus was only observed in

erythroid cells. LCRs for T-cell specific gene expression have also been found (Greaves *et al*, 1989). This approach allows the use of non-specific strategies (such as amphotropic retroviruses or liposomes) for gene delivery.

The second approach, transductional targeting, involves the selective introduction of genes into target cells. In such strategies the vector system targets expression by restricting delivery of the foreign gene to the target cells. The vector systems employed for cell specific gene transfer have included modified retrovirus particles (Etienne-Julan *et al*, 1992), liposomes (Soriano, *et al*, 1983) or polycationic macromolecules (Wu and Wu, 1987).

1.8.1. Receptor-mediated gene delivery.

Receptor-mediated (selective) endocytosis is a natural process which cells use to take up macromolecules such as hormones (epidermal growth factor), low density lipoprotein, and plasma transport proteins (trans-cobalamin, transferrin). The process initially requires the binding of the ligand to its specific cell surface receptor. Once the macromolecules are bound, ligands and receptors tend to cluster into specialised invaginated membrane regions (coated pits). One component of these coated pits is the protein clathrin (Pearse, 1976) which together with associated proteins mediates vesicle formation. Internalisation of the macromolecule then occurs as the clathrin-coated membrane is pinched off as a coated vesicle. After loss of their clathrin coat, vesicles then fuse with and become part of the acidic endosomal compartment. Ligand and receptors can have different fates which include recycling to the cell membrane or degradation in lysosomal compartments. However, the pathways for ligand and receptor processing are highly specific as indicated by the following examples; transferrin (receptor recycled, ligand recycled; Hanover and Dickson, 1985), low density lipoprotein (receptor recycled, ligand

degraded; Basu *et al*, 1981), epidermal growth factor, (receptor degraded, ligand degraded; Stoschek and Carpenter, 1984) and immunoglobulin A (receptor transported, ligand transported; Mostor *et al*, 1984). The internalisation rates of LDL and transferrin are rapid as a result of the clustering of receptors. Other receptor-ligand complexes do not internalise as rapidly but internalise through membrane turnover.

The majority of non-viral vectors systems considered for cell specific gene transfer have consisted of receptor ligands linked to a polycationic DNA binding moiety (Findeis *et al*, 1993). In these vector based strategies the ligand domain permits interaction with cell surface receptors that are constituents of the cellular internalisation pathways (Batra *et al*, 1994) whilst the polycationic polymer condenses DNA to give a particulate system (Wagner *et al*, 1991). In this way, the cellular entry of DNA is subject to the endocytic process described above.

1.8.2. Ligands for targeting polycation-DNA complexes.

The polypeptide based conjugate vectors which have been developed to deliver DNA via the receptor-mediated endocytosis pathway are listed in Table 1.2. However, only those based on asialorosomucoid and cell-selective antibodies are genuine targeting systems as they interact with binding sites expressed on a restricted population of cells. The possibility of targeting with other receptor ligands, such as transferrin, is based on observations which show the overexpression of receptors in certain disease states (eg. transferrin in breast cancer; Faulk *et al*, 1980). However, transferrin receptors are expressed by most cells. Transferrin and asialorosomucoid have been studied widely for the *in vitro* and *in vivo* targeting of polycation complexes. Their use is described in detail in section 1.8.2.1. and 1.8.2.2.

Table 1.2. Proposed strategies for the targeting of polycation-DNA complexes.

Ligand	Receptor	Reference
Asialorosomucoid	galactose specific	Wu *et al*, (1987); Cristiano *et al*,(1993)
Galactose	galactose specific	Perales *et al*, (1994); Chen *et al*, (1994)
Transferrin	transferrin	Wagner *et al*, (1990)
Lactose	membrane lectins	Midoux *et al*, (1993)
Insulin	insulin	Rosenkranz *et al*, (1992)
Lectins	glycolipids/glycoproteins	Batra *et al*, (1994)
Antibodies	cell surface epitopes	Chen *et al*, (1994); Rojanaskul *et al*, (1994)
Folic acid	folate receptor	Gottschalk *et al*, (1994)

1.8.2.1. Asialorosomucoid.

The first method described for the cell specific delivery of DNA consisted of complexing DNA to asialorosomucoid (AsOR) using poly-L-lysine (Wu and Wu, 1987). In these experiments two AsOR-polylysine conjugate molecules were adsorbed per pSV2CAT plasmid, which produced a system with approximately 10 ligand molecules per plasmid. These complexes were taken up selectively in the human hepatoma cell line HepG2, which expresses the asialoglycoprotein receptor; this receptor binds and internalises galactose-terminal (asialo-)glycoproteins. Furthermore, Wu and Wu (1988a), showed that if an excess of ASOR was included in the transfection medium with the targeted polylysine-pSV2 CAT complex it was possible to competitively inhibit the expression of chloramphenicol acetyl transferase.

These systems have also been shown to be capable of delivering DNA *in vivo* (1988b). Following intravenous administration, free AsOR and the AsOR-polylysine-pSV2CAT complexes were taken up preferentially by the liver, presumably by an asialoglycoprotein receptor-mediated process. In studies with a similar system, southern blot analysis in combination with cell isolation techniques, revealed >80% of the DNA-carrier complex internalised by the liver was endocytosed by hepatocytes (Chowdury *et al*, 1993). Therefore, a large proportion of the delivery systems avoided uptake by the phagocytic Kuppffer cells. Gene expression *in vivo* using systems of this type was transient with peak expression of chloramphenicol acetyl transferase seen after 96 hours (Wu and Wu, 1988b). However, the therapeutic potential of DNA vectors based on this technology may be limited as on repetitive administration of these systems to mice, antibodies against both the AsOR-polylysine adduct and DNA complexes have been observed (Stankovics *et al*, 1994).

1.8.2.2. Transferrin.

Wagner *et al*, (1990) have developed a polycation-plasmid DNA delivery system which achieves cellular internalisation through transferrin. The 80 kDa glycoprotein transferrin mediates the uptake of iron into eukaryotic cells through a dimeric transmembrane receptor. The attraction of this pathway for delivery of DNA is that the native ligand is rapidly taken into cells; in erythroblasts as many as 10^4 transferrin molecules are taken up each minute by the $>10^5$ receptors on each cell (Huebers and Finch, 1987). However, this strategy is not likely to achieve selective uptake as receptors for this endogenous ligand are also expressed on proliferating tissues (e.g. bone marrow, intestinal epithelium, epidermis) and some non-proliferating cells (hepatocytes, tissue macrophages, pituitary cells, pancreas islet cells) (Wagner *et al*, 1994a). The value of the transferrin system may be as a generic delivery system, provided that escape from the endosome can be achieved.

Birnstiel and colleagues have used delivery systems of this type for transferrin-dependent receptor-mediated delivery and expression of marker genes in several cell lines in culture (Zenke *et al*, 1990; Cotten *et al*, 1990). Importantly, these workers also demonstrated that the size of the DNA constructs which can be delivered by these systems was in excess of that which could be incorporated into the genome of viral particles. Using this technology, Cotten *et al*, (1992) efficiently transferred a 48-kilobase cosmid into HeLa cells; subsequent expression of the luciferase reporter gene encoded in 48 kbp cosmid was equivalent to that seen on delivery of this gene in an 8 kbp plasmid. More recently transferrin-polylysine-DNA modified with adenovirus particles have been used for the genetic modification of cells in situ (Gao *et al*, 1993). In these studies transient expression of β-galactosidase was seen in the airway epithelial cells of cotten rats for up to 7 days.

1.9. Targeting of bioactive materials using α-melanocyte stimulating hormone (α-MSH)

Mammalian α-MSH (Ac-Ser^{1}-Tyr^{2}-Ser^{3}-Met^{4}-Glu^{5}-His^{6}-Phe^{7}-Arg^{8}-Trp^{9}-Gly^{10}-Lys^{11}-Pro^{12}-Val^{13}-NH_2) is one of a group of structurally related melanocortins that derive from the same precursor molecule, pro-opiomelanocortin (POMC). The sequence of this tridecapeptide was first published in 1957 (Harris and Lerner). In melanocytes and melanoma cells the biological actions of the melanocortins are mediated through the G-protein coupled MC1 receptor (for a review see Eberle, 1988). Significantly, with regard to the use of these melanotropic peptide hormones as targeting moieties, it has now been established that internalisation of the receptor-ligand complex proceeds almost immediately after the binding to the receptor. It is believed that, once in the cell, the hormone is then processed through the endosomal/lysosomal trafficking pathway with degradation of the complex in the lysosome (Adams, 1993).

Several published studies have attempted to employ MSH peptides for the selective delivery of imaging agents, cytotoxic drugs or toxins to melanoma cells. In a recent study Bard *et al*, (1990) used a chelating derivative of α-MSH, bisMSH-DTPA (diethylenamine pentaacetic acid) labelled with ^{111}In for the *in vivo* imaging of malignant melanoma. In this study, tumour associated radioactivity was significantly higher than in other tissues. Conjugates of α-MSH containing melphalan have also been shown to specifically bind to the MSH receptor in a human melanoma cell line. Although the cytotoxicity of these conjugates was less than the free drug alone, it was cell specific, and the effect could be inhibited by excess α-MSH (Morandini *et al*, 1994).

The binding activity of α-MSH has been increased by modifying the native amino acid sequence. Sawyer *et al*, (1980) synthesised [Nle^{4},D-Phe^{7}]α-MSH, which has proved to be a highly potent analog of α-MSH exhibiting prolonged activity. The

binding affinity of this peptide at the MC1 receptor in cultured B16 melanoma cells was subsequently shown to be approximately 10-fold greater than the native peptide (Sahm *et al*, 1994), The more potent and prolonged activity of this peptide is suggested to be due to higher affinity binding to the receptor and a higher stability towards degradation and oxidation. [Nle^4,D-Phe^7]α-MSH analogues should therefore be better suited to the targeting of drugs than the native peptide.

1.10. Lectins for targeting.

Lectins are a group of diverse proteinaceous substances which are characterised by their ability to recognise specific glycoconjugates (glycoproteins/glycolipids) on the surface of cells (Liener *et al*, 1986). This property of lectins has been used to determine phenotypic variants in tissues on the basis of the differential expression of surface carbohydrates (Shimamoto *et al*, 1987). In addition to specific binding it is also apparent that lectins can mediate endocytosis (Roche *et al*, 1983; Roche *et al*, 1990). Lectins have been employed to selectively direct the delivery of toxins (Gilliland *et al*, 1978), chemotherapeutic agents (Lin *et al*, 1981), and polypeptide gene delivery systems (Batra *et al*, 1994).

In the first study a disulphide linked conjugate of concanvalin A (Con A) and fragment A from diptheria toxin was synthesised. The conjugate inhibited protein synthesis in HeLa, CHO or SV3T3 cells in culture, being 100- to 1000-fold more active than the free toxin. This activity was inhibited by ConA and α-methylmannoside. Of more relevance to this project is the possibility of using lectins to target polycation gene delivery systems. Batra *et al*, (1994) showed that the efficiency of gene transfer to Lewis lung carcinoma cells in culture could be increased by employing molecular conjugates containing concanavalin A. Other lectins including wheat germ agglutinin and soybean agglutinin were tested but failed to faciliate gene delivery indicating a degree of specificity.

1.11. Enhancement of gene transfer efficiency using adenovirus and synthetic peptides.

Approaches to increasing the low levels of gene transfer seen in many cell types with ligand-polycation-DNA complexes have focused on the use of mechanisms to enhance the release of particles from the endosome/lysosome compartments. Escape from the endosome has been shown in several studies to be the limiting step in gene expression (Zenke *et al*, 1990; Cristiano *et al*, 1993). The initial strategy employed was the use of the lysosomotropic agent chloroquine which increases the pH of intracellular vesicles and therby inhibits nuclease action (Luthmann and Magnusson, 1983) though the enhancement of gene expression may depend on the osmotic disruption of the endosome. However, the toxicity of chloroquine and related compounds, at the concentrations required to effect endosmolysis, would limit the use of this technique in living organisms (Cotten *et al*, 1990). More recently, this group has used inactivated adenovirus particles to augment delivery of transferrin-polycation complexes both *in vitro* and *in vivo* (Curiel *et al*, 1991; Wagner *et al*, 1992a; Gao *et al*, 1993); this technique has been reviewed comprehensively by Curiel, (1994). The co-internalisation of virus particles and complexes serves to release the DNA-delivery system from the endosomes by virus mediated disruption of the vesicle membrane. This technique has been reported to result in expression of a reporter gene by 100% of cells in culture (Wagner *et al*, 1992a).

More recently this technology has been developed by the replacement of virus particles with fusogenic peptides derived from the influenza virus haemagglutinin protein (HA) (Wagner *et al*, 1992b; Midoux *et al*, 1993). HA produces membrane disruption in the low pH environment of the endosome. Importantly for targeting, this strategy avoids the non-specific binding of viral coat proteins. Cell surface receptors for adenovirus are endogenous on many cell types and promiscuously expressed (Stratford-Perricaudet *et al*, 1992).

1.12. Origin and aims of this study.

Several techniques are available to deliver foreign genes to eukaryotic cells *in vitro*. However, many of these methods are unsuitable for *in vivo* gene therapy as they compromise cell viability or cannot be targeted to a specific cell type. Polycationic polypeptides have previously been investigated as potential carrier systems for the transfer of plasmid DNA to eukaryotic cells and more recently systems modified with cell surface receptor ligands have demonstrated selective gene transfer to cells *in vitro* and *in vivo*. Conceptually, model systems of this type represent replication deficient, membrane-free, synthetic viruses that can encapsulate large sequences of DNA (Wagner *et al*, 1992a).

The initial aim of this project is to use a cell specific recognition system to achieve a level of selective delivery to melanoma cells *in vitro*; our model being the binding of α-MSH and analogs to melanocortin receptors. Previous attempts to target genes to these cells have been through the less specific technique of transferrinfection (Plank *et al*, 1994; Wagner *et al*, 1994b). A general method using the lectin concanavalin A will also be tested. Molecular conjugate vectors are to be formed by linking ligands to DNA through poly-L-lysine modified with the 60 kDa biotin-binding protein streptavidin. This technology provides a universal method for the attachment of biotinylated compounds. Histone H1 and alanine-lysine graft co-polymers are to be investigated as alternative carriers to poly-L-lysine.

In developing cationic polypeptide gene delivery systems it is thought important to systematically investigate the formulation factors such as polypeptide-to-nucleic acid ratio and carrier molecular weight which have previously been suggested to affect delivery efficiency. Moreover, it is recognised these factors are intrinsically linked to the physicochemical properties of complexes. From measurements of DNA condensation, complex morphology and electrostatic charge it is hoped to gain

further understanding of the mechanisms underlying polycation-mediated gene transfer. The *in vitro* stability of polycation-DNA complexes are also to be assessed.

Chapter 2

Complexation of Deoxyribonucleic acid using cationic polypeptides.

It is now widely recognised that cationic polypeptides interact spontaneously with negatively charged nucleic acids resulting in the formation of particulate systems (Felgner, 1990). These complexes have recently been used to facilitate gene transfer to eukaryotic cells. The work detailed in this chapter describes the formulation of polycation-DNA complexes to be used in subsequent gene transfer experiments. Systems formulated using poly-L-lysine, histone H1 and a series of alanine substituted polylysine derivatives are described. Formation of complexes between these cationic polypeptides and DNA was investigated using qualitative and quantitative assays based on the exclusion of the fluorescent probe ethidium bromide.

2.1. Deoxyribonucleic Acid (DNA).

Linear double stranded calf thymus DNA was purchased as the sodium salt (Sigma). Prior to use DNA was dissolved in TE buffer (pH 8.0) and stored at -20°C. The majority of DNA fragments were in the range 564-21,224 base pairs. Size analysis was performed by agarose gel electrophoresis (section 2.1.6.).

Gene transfer was monitored using a 7.8 kbp plasmid construct, pRSV*lacZ*, in which the *Eschericia coli* β-galactosidase reporter gene is under the control of the rous sarcoma virus long terminal promoter/enhancer sequence. To facilitate selection this plasmid also encoded ampicillin resistance. pRSV*lacZ* was obtained from Dr. D. Ogllvie (Zeneca Pharmaceuticals, Alderley Edge, Chesire, U.K.). Plasmid DNA was propagated in *E.coli* XL1-Blue (Stratagene), isolated by the

alkaline lysis technique (Birnboim and Doly, 1979) and purified by anion exchange chromatography (see section 2.1.4.). *E.coli* XL1-Blue shows a phenotypic resistance to tetracycline (Bullock *et al*, 1987). Plasmid containing bacteria were grown in LB medium supplemented with ampicillin (50 μg/ml) and tetracycline (12.5 μg/ml).

2.1.1. Preparation of competent E.coli XL1-Blue cells.

E.coli cells were transformed using a variation of the heat shock technique described by Cohen *et al*, (1972). Competent cells were prepared as follows: An overnight culture of *E.coli* XL1-Blue cells was diluted 1:10 and grown to an OD_{600} of 0.2-0.3 in 40 ml LB medium. Cells were then chilled on ice and centrifuged at 4000g for 10 minutes at 4°C. The medium was removed and the cells resuspended in 5 ml ice cold 0.1M $CaCl_2$. The cell suspension was stored on ice for 30 minutes before the cells were re-centrifuged and suspended in 1.0 ml of 0.1M $CaCl_2$. Competent cells were stored overnight at 4°C before use.

2.1.2. Measurement of bacterial growth in liquid media.

The number of cells present in culture was estimated by spectrophotometric measurement at 600 nm (Milton Roy Spectronic 601). At low density, cell concentration is directly proportional to optical density (in the range OD = 0.03-0.3), where optical density is defined according to the Beer-Lambert law. An OD of 1.0 at 600 nm corresponds to approximately 1 $x10^9$ cells/ml.

2.1.3. Transformation of E.coli XL-1 Blue cells by heat shock.

Three hundred microlitres of the competent cell suspension was transferred to a thin walled glass tube. pRSVlacZ DNA (3 μg) was added to the cells and the contents of the tube mixed. The cells were heat shocked by placing the tube in a water bath, pre-heated to 50°C, for 45 seconds and then chilled on ice for 2 minutes.

Following heat shock, cells were allowed to recover in 1 ml SOC medium at 37°C with shaking for 1 hour (to allow expression of plasmid-coded antibiotic resistance). Transformed cells were selected by spreading onto LB agar plates containing ampicillin (50 µg/ml) and tetracycline (12.5 µg/ml). In a control transformation, antibiotic resistance was confirmed as plasmid mediated by subjecting cells to heat shock in the absence of the recombinant DNA.

2.1.4. Large scale isolation and purification of plasmid DNA.

Bacterial suspension was transferred to four 50 ml tubes and centrifuged at 4,000 rpm for 15 minutes. The supernatant was then carefully decanted from each tube, so as not to disturb the pellet. This step was repeated for the remainder of the 500 ml culture. The composition of all buffers used in this procedure is described in appendix 1. Each pellet was resuspended in 2.5 ml buffer P1, using a sterile Pasteur pipette, before being pooled in an ultra-centrifuge tube. Ten millilitres of buffer P2 was added and the tube contents mixed by gently inverting five times. After four minutes 10 ml of buffer P3 was added to the cell lysate. The tube was placed on ice for 30 minutes and then centrifuged at 25,000 rpm at 4°C for 45 minutes in a Beckman Ti45 rotor. The supernatant was removed promptly and applied onto a single-use modified silica anion exchange purification column (Qiagen). Prior to use the column was pre-equilibrated with 10.0 ml of buffer QBT. The column was then washed twice with 30 ml of buffer QC. Plasmid DNA was eluted using 15 ml of buffer QBT and precipitated by the addition of 10.5 ml of isopropanol. The tube contents were gently mixed and centrifuged at 4°C for 45 minutes at 25,000 rpm. Pelleted DNA was washed with 15 ml of ice-cold 70 % ethanol and repelleted by centrifugation. Following aspiration of the supernatant, residual ethanol was removed from the pellet by air drying at room temperature for 10 minutes. Plasmid DNA was resuspended in TE buffer (pH 8.0) and stored for up to 3 months at -20°C. Each isolation yielded approximately 500 µg of purified plasmid DNA.

2.1.5. Spectrophotometric determination of nucleic acid concentration.

The quantity and purity of DNA was determined using triplicate spectrophotometric readings at 260 nm and 280 nm (Milton Roy Spectronic 601). Measurements were made against a blank sample of TE buffer (pH 8.0). An OD of 1.0 at 260 nm corresponds to a solution of 50 μg/ml for double stranded DNA (Sambrook *et al*, 1989). The purity of the DNA solution was determined by measurement of the ratio of absorbances at 260 nm and 280 nm. Protein contamination of DNA solutions would reduce this ratio to below 1.8. All plasmid DNA samples used in transfection and complexation experiments had an A_{260}:A_{280} ratio greater than 1.8.

2.1.6. Preparation of a horizontal agarose gel.

Agarose gels were cast and run in Bio-Rad DNA sub-cells. Ultrapure agarose (Bio-Rad) was dissolved in TAE buffer at 100°C to give a gel of the appropriate concentration. The solution was allowed to cool to 60°C, made up to weight with water, and ethidium bromide added to 0.5 μg/ml. The gel was poured and allowed to set at room temperature for 45 minutes. Once set, gels were submersed in TAE buffer. DNA samples were mixed with 0.2 volumes of 5X DNA loading buffer and electrophoresis carried out at 70 V until the tracking dye reached the end of the gel. DNA bands were visualised on a UV light box. Photographs were taken using Polaroid 55 film in a Polaroid camera. Negatives were prepared by washing in an 18% $^{w}/_{v}$ solution of sodium sulphite and then rinsing with water.

2.1.7. Digestion of DNA with restriction endonucleases.

DNA restrictions were performed in accordance with manufacturer's instructions. For complete plasmid digestion, reaction mixtures were allowed to incubate at 37°C for 60 minutes. Partial digests are described in section 2.1.8.

2.1.8. Partial digestion of pRSVlacZ with XbaI.

To 1 µg of plasmid DNA were added 5 µl of 5X reaction buffer (Sigma), 2.5 µl BSA solution (2 mg/ml) and 1 µl *XbaI* (Sigma 20 units/µl). The total volume was made up to 50 µl with sterile water and the reaction mixture mixed by pipetting and incubated at 4°C. At 5 minute time intervals reactions were stopped by the addition of 7 µl DNA loading/reaction stop buffer. Samples were stored on ice until needed. After the final samples had been removed, all samples were run on a 1.0% agarose gel.

2.1.9. Scanning densitometry.

Gel photographs (negative) were scanned using a Bio-Rad scanning densitometer model GS-570. Analysis of the digitised images was performed using Molecular Analyst software package. Quantification of bands was determined using Volume Analysis software (Bio-Rad).

2.2. Cationic polymers.

Cationic polymers used in this study are described below and the physical characteristics summarised in Table 2.1. and 2.2. Polymer solutions were prepared under clean laboratory conditions. Hepes buffered saline (HBS: 20 mM Hepes/150 mM NaCl, pH 7.4) or Milliq water was filter sterilised using a 0.2 µm nitrocellulose filter (Sartorious).

2.2.1. Poly-L-lysine.

Poly-L-lysine Hydrobromide samples [average degree of polymerisation (determined by light scattering) = 13, 214 and 859] and Poly-L-lysine Hydrochloride (DPn=127) were obtained from Sigma. All polymers were polydisperse. Prior to use polymers were dissolved in HBS or Milliq water to give a 5.0 mg/ml solution and stored at 4°C. Polymers were stored in rubber capped glass vials (Chromacol).

Table 2.1. Physical characteristics of poly-L-lysine polymers. Data were supplied by the manufacturer.

Polymer	DPn	Mol. Wt. (free polymer)	Mol. Wt (salt)	Mw/Mn
Poly-L-lysine•HBr	13	1684	2736	N/A
Poly-L-lysine•HCl	127	16300	21000	1.25
Poly-L-lysine•HBr	219	28000	45800	1.1
Poly-L-lysine•HBr	859	110100	179600	1.1

N/A=Not available.

2.2.2. Histone H1.

Calf thymus histone H1 (21 kDa) was supplied as 1 mg of lyophilised solid (Calbiochem). The solid was reconstituted with HBS to give a 1 mg/ml solution and stored in 100 µl aliquots at -70°C in 1.5 ml microcentrifuge tubes. Aliquots of histone H1 were not re-frozen once defrosted.

2.2.3. Alanine-lysine graft co-polymers.

Alanine-lysine graft co-polymers (AK 100, AK200, and AK500) were a gift from Professor Ferenc Hudecz (Eotyos University, Budapest, Hungary). The physical properties of these graft co-polymers are described in Table 2.2 and their chemical structure illustrated in Figure 2.1. Polymers were supplied as a 100 mg/ml solution in water and diluted with HBS to give a 2 mg/ml solution. Polymers were stored at -20°C in 0.5 ml microcentrifuge tubes.

Table 2.2. Physical characteristics of alanine-lysine graft co-polymers.

Polymer	DPn	Mol.wt.	Ala:Lys Ratio	Mol.wt. (Monomer unit)
AK100	66	28200	4.2 : 1.0	426.8
AK200	280	89700	2.7 : 1.0	320.2
AK500	650	221800	3.0 : 1.0	341.2

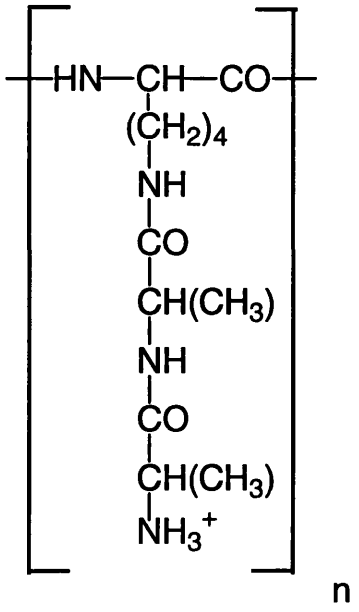


Figure 2.1. Chemical structure of alanine-lysine graft co-polymers. The α-amino group of these polymers provides the cationic charge for interaction with DNA. Monomer units varied in the number of alanine residues grafted to the each lysine residue.

2.3. Calculation of Polymer-to-DNA charge ratio.

Complexation of pRSVlacZ and chromosomal DNA were determined as a function of both the mass of cationic polypeptide added and the positive to negative charge ratio.

$$\text{Charge Ratio } (+/-) = \frac{\text{Total number Positive charges (mols)}}{\text{Total number Negative charges (mols)}}$$

2.3.1. DNA.

The calculation of negative charge provided by DNA was determined on the basis that each nucleotide in the DNA sequence is associated with a single negative charge. A mean value of 330 was calculated for the molecular weight of a monophosphorylated nucleotide from data given by Sambrook *et al,* (1989) for dAMP, dCMP, dGMP and dTMP.

2.3.2. Poly-L-lysine homopolymers.

Positive charge provided by synthetic poly-L-lysine homopolymers was calculated using the molecular weight and DPn determined by the manufacturer. Compensation was made where polymers were supplied in salt form.

2.3.3. Alanine-lysine graft co-polymers.

Using the alanine:lysine ratio described in Table 2.2. the effective molecular weight of each monomer unit, with which one charge is associated, was calculated. From this value the charge provided by a polymer molecule was then calculated. Complete ionisation of α-amino terminal groups was assumed.

2.3.4. Histone H1.

Calculation of charge provided by histone H1 was based on the presence of basic lysine and arginine residues in the polypeptide sequence. Amino-acid analysis of calf thymus histone H1 was performed by Bradbury *et al*, (1975a).

2.4. Formation of polycation-DNA complexes

Polycation-DNA complexes were prepared by mixing dilute solutions of the polypeptide with DNA. Stock solutions of polylysine polymers were diluted to a concentration of 78 μg/ml (free polylysine). For experiments using histone H1 and alanine-lysine graft co-polymers, working concentrations of 200 μg/ml were produced. All dilutions were prepared in HBS. DNA solutions were prepared by diluting 6 μg pRSVlacZ or calf thymus DNA to 250 μl (24 μg/ml) in a sterile 1.5ml microcentrifuge tube. Varying masses of polypeptide were then added to individual microcentrifuge tubes and diluted to a total volume of 250 μl. The mass range of polypeptide used in complexation reactions is described in Table 2.3. Each polypeptide solution (250 μl) was then added dropwise to 250 μl of DNA solution (24 μg/ml) while vortex mixing. Complexation was allowed to proceed at ambient temperature for 30 minutes before analysis by gel electrophoresis or spectrofluorimetry.

Table 2.3. Mass and charge range of polypeptides used in complexation reactions.

Polymer	pLL (13)	pLL (127)	pLL (214)	pLL (859)
Mass Range (μg)	0 -14.4	0-6.0	0-6.0	0-4.8
Charge Range(mols)	$0\text{-}1.1\times10^{-7}$	$0\text{-}4.7\times10^{-8}$	$0\text{-}4.7\times10^{-8}$	$0\text{-}3.7\times10^{-8}$
Polymer	AK100	AK200	AK500	Histone H1
Mass Range (μg)	0-15.6	0-13.8	0-12.6	0-2.3
Charge Range (mols)	$0\text{-}3.7\times10^{-8}$	$0\text{-}4.3\times10^{-8}$	$0\text{-}3.7\times10^{-8}$	$0\text{-}3.3\times10^{-8}$

2.5. Ethidium bromide as a fluorescent probe.

Ethidium bromide acts as a fluorescent probe following intercalation between DNA base pairs. Ultraviolet radiation at 254 nm is absorbed by DNA and transmitted to the dye with energy re-emitted at 590 nm in the red-orange region of the visible spectrum (Sambrook *et al*, 1989). The fluorescent yield of the dye on intercalation is greater than for the dye in free solution. This effective change in intensity allows nucleic acid to be distinguished against background in agarose gels and also allows quantification of DNA using spectrofluorimetry.

2.5.1. Analysis of DNA complexation by fluorescence spectrophotometry.

Quantitative analysis of complexation was determined using a method based on that described by Gershon *et al*, (1993). Polymer-DNA complexes containing 6μg pRSVlacZ in 0.5 ml were formed as described in section 2.4. Following incubation at room temperature for 30 minutes, 500 μl of complex suspension was added to 2.5 ml 150 mM NaCl. Immediately prior to analysis, 3 μl ethidium bromide (0.5 mg/ml) was added to the various DNA-polymer mixtures (1:5 molar ratio of probe to nucleotides). Each sample was then mixed by vortexing and fluorescence monitored (λ_{ex} = 260 nm, λ_{em} = 600 nm; 1 cm light path cell) using a Shimadzu RF-540 spectrofluorophotometer, with 5 nm excitation and emission slits. Complexation of DNA produces a reduction in fluorescence as intercalation of ethidium bromide between DNA base pairs is prevented. The degree of complexation was measured as a decrease in fluorescence relative to the control, which contained 6 μg uncomplexed plasmid DNA. Fluorescence of the control sample was normalised to 100%.

2.5.2. Gel mobility shift assay.

Gel mobility shift assays complemented quantitative spectrofluorometric methods for determining complexation of plasmid DNA. Complexes containing increasing

quantities of polypeptide were prepared as in section 2.4. Immediately after formation, 40 µl of each complex suspension was removed and 10 µl of 5X loading buffer added. A 10 µl aliquot from each preparation, containing 100 ng plasmid DNA, was then resolved by electrophoresis through a 1% agarose gel at a constant voltage (80V) for 75 minutes. Gels contained ethidium bromide (0.5 µg/ml) for visualisation of DNA. Following electrophoresis, gels were viewed and photographed as described in section 2.1.6.

RESULTS

2.6. Analysis of DNA for use in transfection.

Following isolation and purification, plasmid pRSVlacZ was analysed by gel electrophoresis to determine the suitability for use in transfection experiments. A representative gel is shown in Figure 2.2. Plasmid-DNA prepared by alkaline lysis (Fig. 2.2.; Lane 5) were routinely shown to be free from contamination with RNA or chromosomal DNA. Bands were identified by partial digestion using *Xba*I (Fig. 2.2.; Lane 2 and 3). During electrophoresis DNA migrates from the origin of the gel toward the anode (Bottom of gel). From the bottom of the gel, bands corresponded to supercoiled, nicked open-circle, and linear forms of plasmid DNA respectively. Digestion of pRSVlacZ using 20 units of restriction enzyme at 37°C for 30 minutes (Fig 2.2.; Lane 4) showed a single band of 7.8 kilobases. Band densities were determined by scanning densitometry volume analysis (as described in section 2.1.9.) with appropriate corrections for supercoiled DNA which appears underrepresented on visual examination; the conformation of supercoiled DNA reduces ethidium bromide intercalation by 30% (Koch *et al*, 1993). Samples used in transfection experiments routinely contained greater than 85% supercoiled DNA.

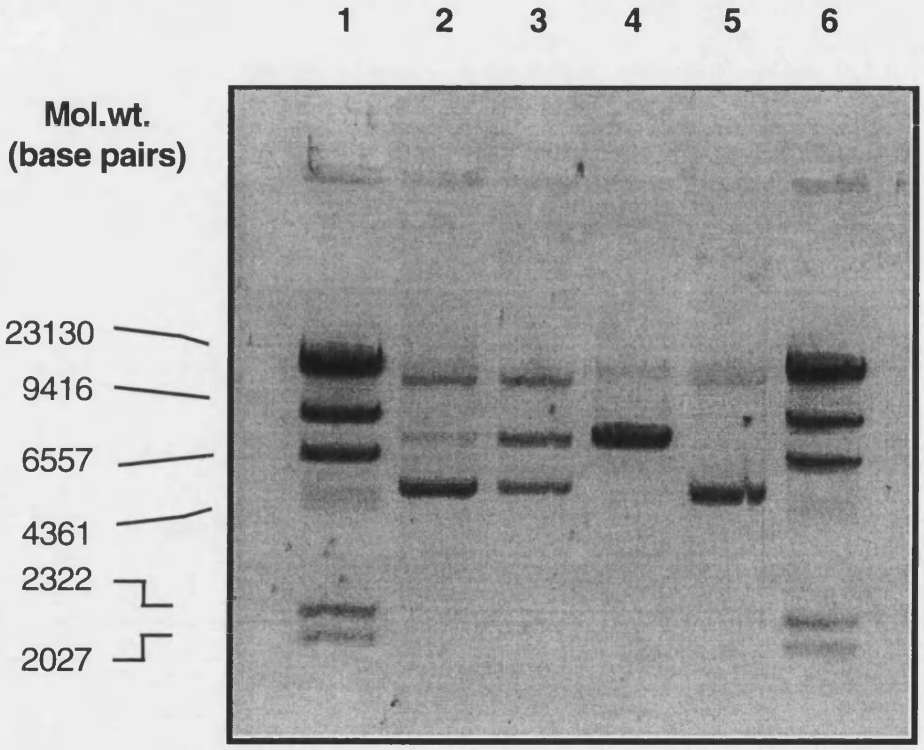


Figure 2.2. Agarose gel electrophoresis of pRSVlacZ. **Lane 1** and **6**, *Hind*III-cut λ DNA; **Lane 2**, pRSVlacZ (*Xba*I, 10 minutes, 4°C); **Lane 3**, pRSVlacZ (*Xba*I, 20 minutes, 4°C); **Lane 4**, pRSVlacZ (*Xba*I, 60 minutes, 37°C); **Lane 5**, pRSVlacZ (undigested).

2.7. Complexation of plasmid DNA using poly-L-lysine.

Figure 2.3. demonstrates the influence of polymer chain length on the complexation of pRSVlacZ-DNA by a series of polylysine polymers. In these experiments increasing quantities of polymer (described in Table 2.3.) were mixed

with 6 μg DNA. For pLL(127), pLL(214) and pLL(859) fluorescence intensity was reduced on the addition of cationic polypeptides in a dose dependent manner. The number of molecules required to be added to DNA in order to produce fluorescence quenching was inversely related to the DPn (Fig. 2.3.a). Therefore, to effect fluorescence quenching with pLL(127) 6.8 times the number of pLL(859) molecules was required. The specific polypeptide-to-DNA mass ratio at which fluorescence intensity was reduced to background levels (Fig. 2.3.b; Polymer:DNA = 0.4:1) corresponded to a charge ratio of 1.1 (Fig. 2.3.c). However pLL(13), the smallest polymer tested, appeared to behave anomalously as maximum fluorescence quenching was achieved only on the addition of a two fold mass excess of polymer. The quench curve produced with this polymer was consequently shallower than those seen for pLL(127), pLL(214) and pLL(859). An effective polymer:DNA charge ratio (+/-) at the point of complexation was 5.1. In addition, fluorescence intensity was only reduced to a minimum of 20.6% of the control level.

2.7.1. Gel electrophoresis of Poly-L-lysine-DNA complexes.

The mobility shift assay performed on complexes formed between pLL(214) and pRSVlacZ-DNA is shown in Figure 2.4.a. Based on charge neutralisation, as shown by the reduction of the electrophoretic mobility of DNA, the interaction of poly-L-lysine(214) and DNA could be detected at a mass ratio of 0.1:1 (Fig. 2.4.a). In this sample, both unretarded free DNA, which migrated through the gel at the same rate as pRSVlacZ, and complexed DNA which was retained at the gel origin could be detected. At mass ratios of 0.3:1 and greater, free DNA could not be detected in the gel. Poly-L-lysine labelled with fluorescein-isothiocyanate (Sigma) was used to form complexes with pRSVlacZ for use in uptake studies. Using pLL-FITC(219) complete retardation of pRSVlacZ was achieved at a polymer:DNA mass ratio of 0.4:1 (Fig. 2.4.b).

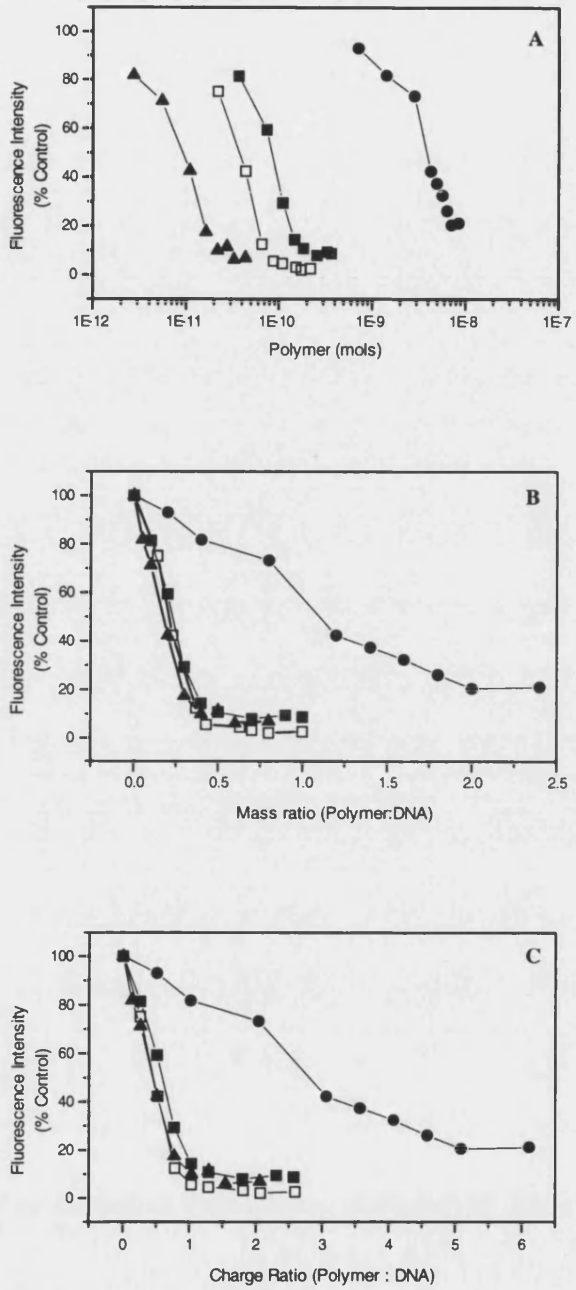
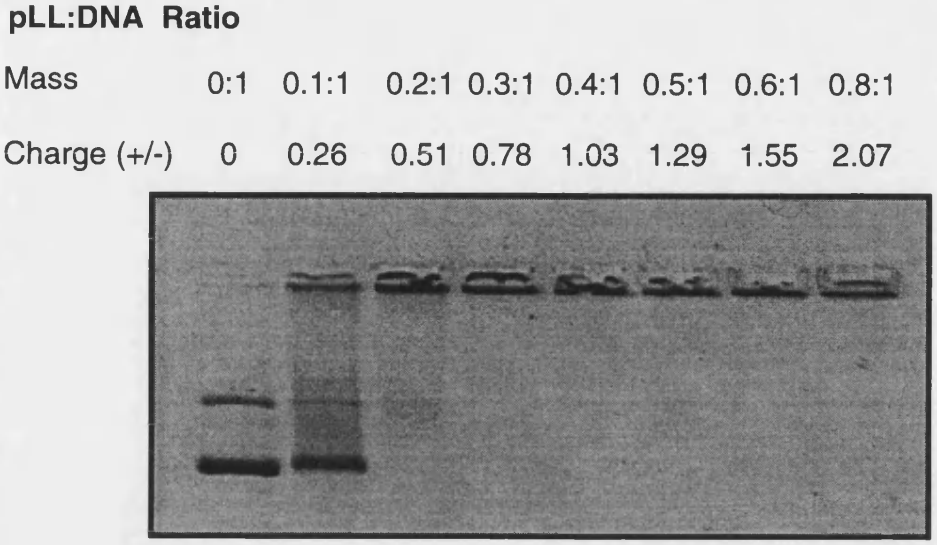


Figure 2.3. Complexation of DNA using poly-L-lysine. pRSVlacZ-DNA was mixed with increasing quantities of (●) pLL(13), (■) pLL(127), (□)pLL 214 and (▲) pLL(859). Data is expressed in terms of the molar quantity of polymer added to DNA (Plate A), polymer:DNA mass ratio (Plate B) and charge ratio (Plate C). Data presented is compiled from representative experiments.

A



B

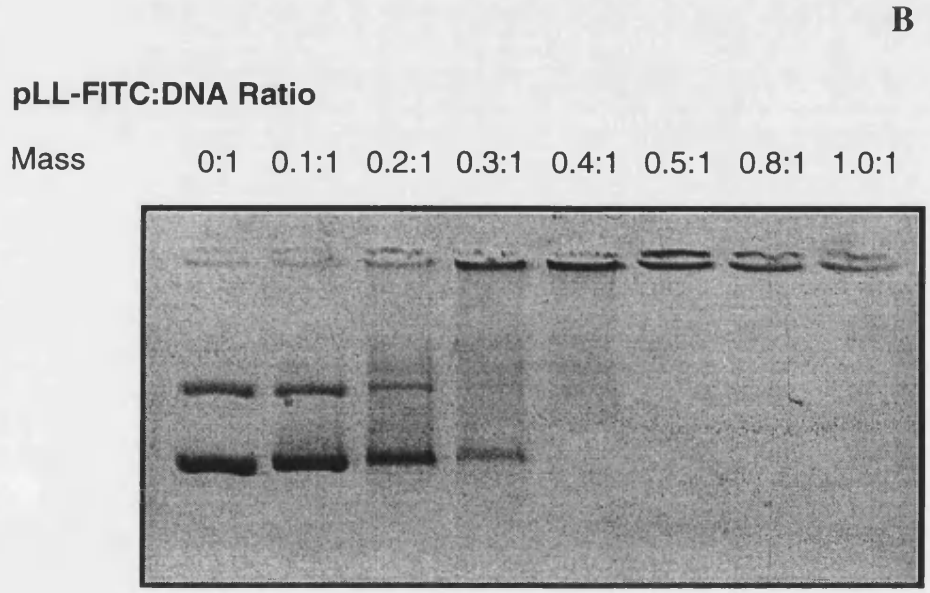


Figure 2.4. Agarose gel electrophoresis analysis of poly-L-lysine-DNA complexes. pRSVlacZ was incubated with increasing amounts of pLL (Plate A) or pLL-FITC (Plate B) and incubated at room temperature for 30 minutes. 0:1 represents pRSVlacZ only; bands represent from bottom to top of gel, plasmid DNA in supercoiled and open circle conformations.

2.7.2. *Influence of DNA conformation and the presence of buffer salts on complexation.*

Measurements made on complexes formed with an equal mass of calf thymus DNA or closed-circular-supercoiled DNA (6 μg) and increasing quantities of pLL(214) showed similar fluorescence patterns (Fig. 2.5.). In each case background levels of fluorescence intensity were reached at a polymer:DNA mass ratio of 0.4:1 which corresponds to a charge ratio of 1.1. Complexes prepared for transfection experiments were usually prepared in HBS. However, to assess if the presence of buffer salts affected the complexation process, systems were prepared in double distilled de-ionised water. Under these conditions the mass of polymer required to induce fluorescence quenching was the same as in the presence of buffer salts (Polymer:DNA; 0.4:1) indicating that at the concentrations routinely used in complex preparation sodium chloride and Hepes did not affect complex formation.

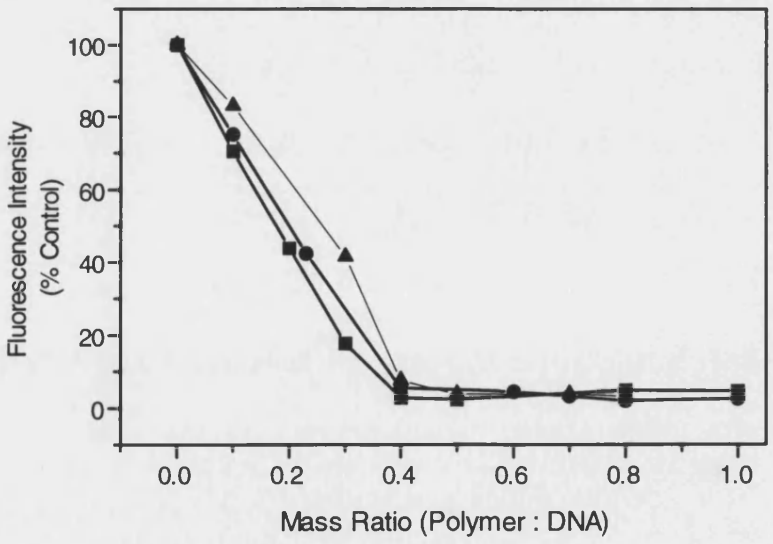


Figure 2.5. Effects of DNA conformation and buffer salts on complexation. Complexes were formed between pLL(214) and 6 μg calf thymus-DNA (■) or pRSVlacZ-DNA (●) in HBS. Effect of buffer salts was assessed by forming complexes between pLL(214) and pRSVlacZ in water (▲).

2.8. Complexation of DNA using novel cationic polypeptides.

Fluorescence quenching was achieved with alanine substituted poly-L-lysine polymers, of 66 (AK100), 280 (AK200) and 650 (AK500) monomer residues, at polymer-to-DNA mass ratios of 2.3, 1.7 and 1.5 respectively (Fig. 2.6.a). The fluorescence quench curve for pLL(214) is also shown. For each of the alanine-lysine heteropolymers the amount of charge required to produce fluorescence quenching was found to be greater than for pLL(214). AK100 and AK200 showed similar fluorescence quench curves with no further quenching observed above a charge ratio of 1.8. In contrast, complete quenching was produced by pLL(214) at a charge ratio of 1.1 (Fig. 2.6.b).

2.9. Analysis of Histone H1-DNA complexes.

Complexation of plasmid-DNA by histone H1 was examined by both spectroflourimetry (Fig. 2.7.) and gel mobility shift assay (Fig. 2.8.). In order to achieve fluorescence quenching with H1, a 1.7 fold mass excess of polypeptide was required to be added to DNA. Fluorescence exhibited at this polypeptide-to-DNA mass ratio was 14.5% of the control value and was not reduced by further increasing the mass excess of polypeptide. In contrast, condensation of DNA using pLL(214) reduced fluorescence intensity to 3% of the control value. The charge ratio at which fluorescence quenching occurred was 1.1 and 1.4 for pLL(214) and H1 respectively (Fig. 2.7.b). Agarose gel electrophoresis (Fig. 2.8.) showed that as the proportion of histone H1 in the samples increased there was a decrease in the electrophoretic mobility of DNA. The movement of H1-DNA complexes formed at a mass ratio of 0.4:1 was retarded, compared to free pRSVlacZ-DNA, but moved freely in the gel and was not retained at the origin. This is in contrast to the complexation of DNA by poly-L-lysine where plasmid molecules existed in either the free or complexed state. Complete localisation of DNA in the well of the gel was achieved at H1:DNA mass ratios >1.0:1.

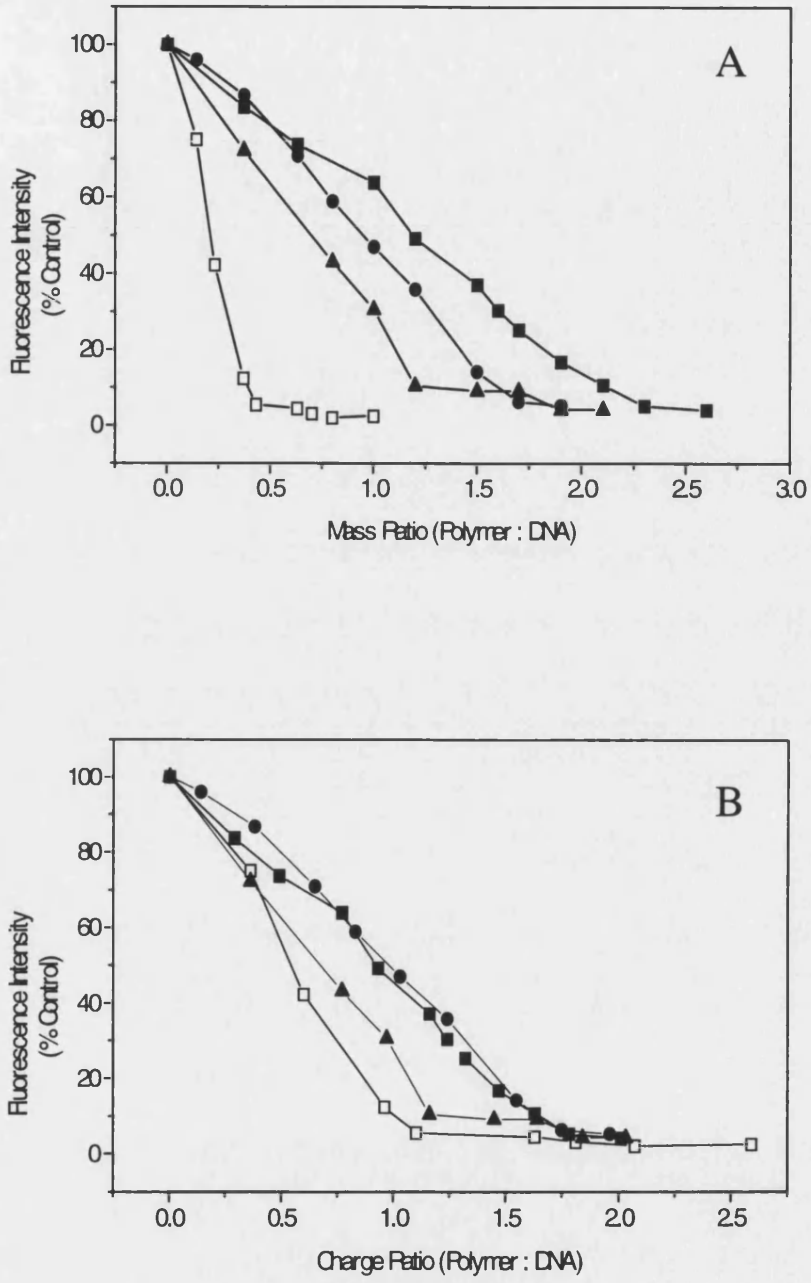


Figure 2.6. Complexation of pRSVlacZ-DNA using alanine-lysine graft co-polymers. Effect of polymer-to-DNA ratio on fluorescence intensity in terms of mass (Plate A) and charge (Plate B) for poly-L-lysine (214) and a series of alanine-lysine graft co-polymers. Complexes were formed with pLL 214(□), AK100(■), AK200(●) and AK 500(▲).

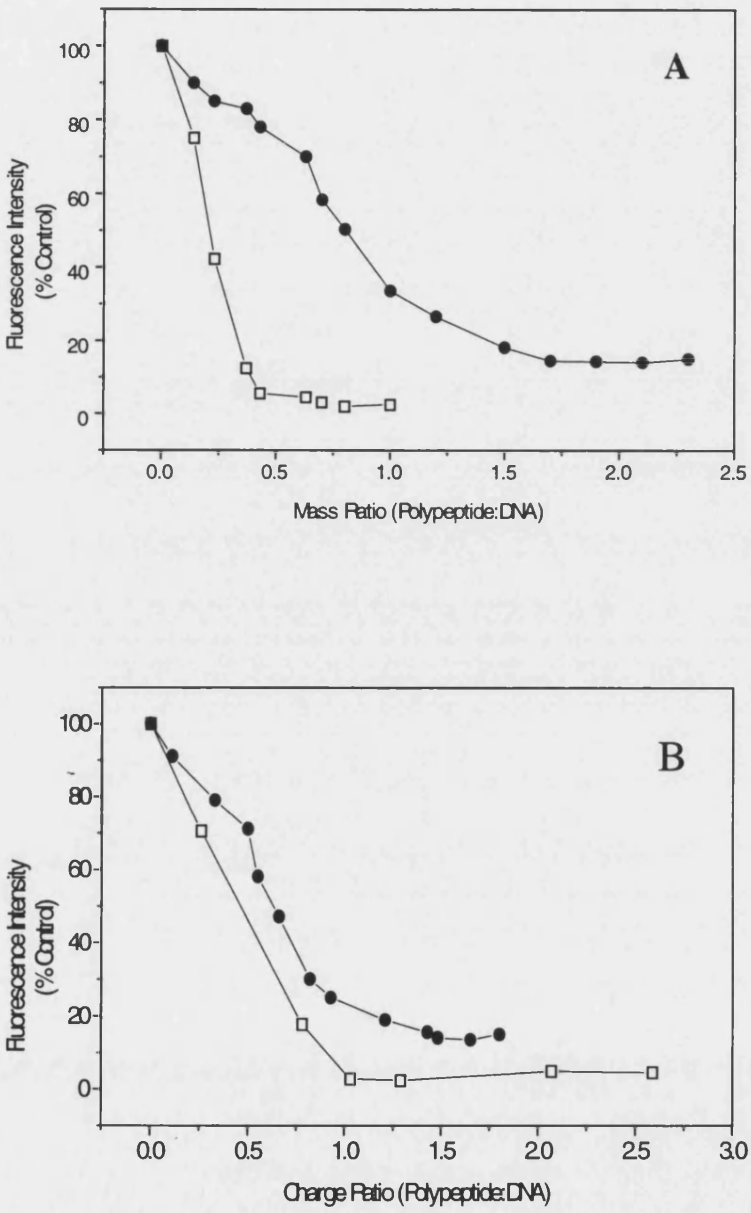


Figure 2.7. Complexation of pRSVlacZ-DNA using histone H1. Effect of Polypeptide-to-DNA to ratio on fluorescence quenching in terms of mass and charge. Complexes were formed using (❑) pLL(214) and histone H1 (●) as described in section 2.4.

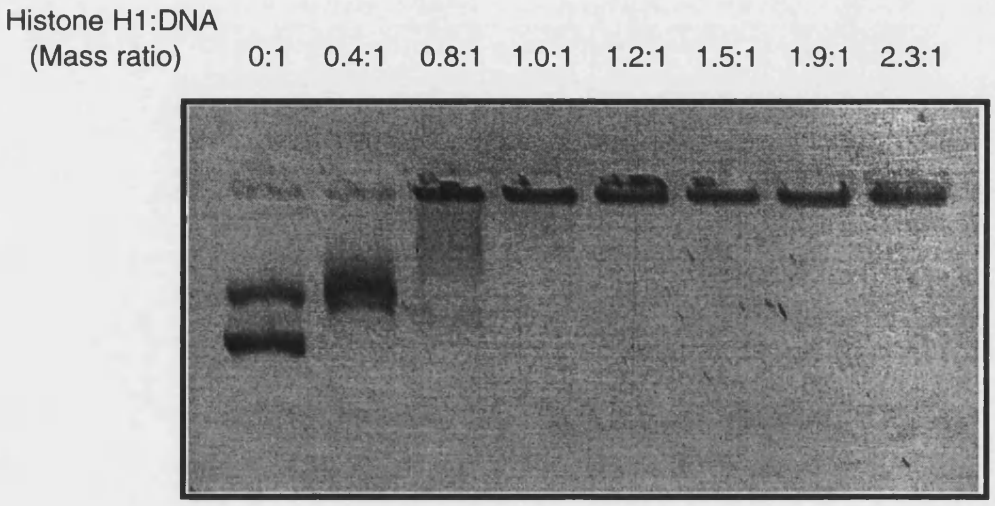


Figure 2.8. Agarose gel electrophoresis of histone H1-pRSVlacZ-DNA complexes. pRSVlacZ was incubated with increasing amounts of H1. 0:1 represents pRSVlacZ only.

2.10. Discussion.

The co-operative interaction of linear DNA and the cationic homopolypeptides polylysine and polyarginnine has previously been described using circular dichroism (Olins *et al*, 1967) and ultracentrifugation (Leng and Felsenfeld, 1966). However, data were not available which detailed the quantity of polymer required to completely complex plasmid-DNA under the conditions to be used for *in vitro* transfection experiments. Analysis of the interaction between plasmid-DNA and cationic polypeptides in these initial studies was therefore motivated by the desire to formulate complexes in a reproducible manner. A basic requirement prior to the development of gene delivery systems of this type was the isolation of plasmid-DNA of high purity.

2.10.1. Preparation of plasmid-DNA for transfection.

Plasmid-DNA used throughout this study was purified by anion-exchange chromatography following isolation by alkaline-lysis and was shown to be free from RNA and contaminating protein. This is important as a critical parameter in the formation of reproducible complexes was thought to be the accurate quantification of DNA. The presence of RNA in plasmid DNA samples would result in the over-estimation of DNA concentration. RNA as a nucleic acid is also capable of interacting with cationic polypeptides and would compete for polypeptide binding.

In addition to purity, observations by Lehmann and Oomen, (1985) indicated that the topology of DNA molecules affects the level of transcription. This study showed the level of chloramphenicol acetyl transferase expression was markedly higher when supercoiled DNA's rather than linear DNA's were transfected into monkey CV-1 cells. Subsequently, Weintraub *et al*, (1986) demonstrated supercoiled DNA to be a more effective template for transcriptions. The influence of plasmid conformation

on gene expression was found to be most pronounced in plasmids, such as pRSVlacZ which contain enhancer sequences; the supercoiling of DNA increasing the efficiency with which bound transcription factors were used. Therefore, maximisation of expression requires delivery of DNA in the supercoiled form. DNA samples used in this study routinely contained greater than 85% of DNA molecules in this conformation.

Purification of plasmid-DNA by anion-exchange column chromatography was also found to be more convenient than the traditional method of caesium chloride gradient centrifugation which requires the labelling of DNA with ethidium bromide. However, Wicks *et al*, (1995) have shown this method does not remove lipopolysacchride (LPS) which has been shown to reduce transfection efficiency (Cotten *et al*, 1994). A chromatographic method for removing LPS contamination has recently been reported (Horn *et al*, 1995).

2.10.2. Complexation of plasmid-DNA using poly-L-lysine.

Analysis of plasmid-DNA complexation using poly-L-lysine of differing degrees of polymerisation demonstrated similar behaviour for polymers of 127, 214 and 859 monomer units. The exclusion of ethidium bromide from poly-L-lysine complexes was independent of the number of polymer molecules in the reaction system. However, for each of the polymers tested complete complexation was achieved at a polypeptide-to-DNA mass ratio of 0.4:1 which equated to a theoretical charge ratio of 1.1. Preparation of complexes in either HBS or water did not affect this process. These data are in accordance with the general theory on DNA complexation which has shown that at a given degree of charge neutralisation, DNA molecules collapse into packed forms in a highly co-operative process (Manning, 1981). The electrostatic interaction of polylysine and DNA has previously been demonstrated to induce this phenomenon with complexation and subsequent condensation of DNA

being significantly affected by the ratio between the two polymers (Carrol, 1972). The extensive ultracentrifugation of systems formed between calf thymus DNA and pLL(100), at lysine:phosphate ratios >1.0, showed DNA was completely incorporated into a fraction with a sedimentation profile different to that of naked DNA (Leng and Felsenfield, 1966). This suggests a transition between a solution state and a colloidal system on complexation. The fluorescence quenching of ethidium bromide observed on incubation with complexes formed at charge ratios >1.1 is therefore indicative of the exclusion of fluorescent probe from collapsed DNA structures. Gershon *et al*, (1993) demonstrated that a similar positive charge density (Liposome:DNA;(+/-) = 1.2) was required to achieve DNA condensation using cationic liposomes (DOTMA/PE, 1:1). The overall positive charge density remained identical when pLL(90) was used to provide partial charge neutralisation in these systems. These data show good correlation with the work of Tsuboi *et al*, (1966) who found the binding of the homopolymer polylysine to DNA to have 1 to 1 stoichiometry (one basic residue per nucleotide).

Complexes formed between pLL(13) and plasmid-DNA, however, require higher lysine:phosphate ratios to induce condensation. This perhaps suggests that small chain length polymers are ineffective at binding or condensing DNA. Complete exclusion of ethidium bromide was only achieved by increasing the concentration of pLL(13) and effectively shifting the equilibrium between bound and free polylysine. The form of these complexes also appears different since at high polypeptide-to-DNA ratios the fluorescence intensity of complexes was only reduced to 20.6% of the control level. This is in contrast to the background levels achieved using polylysines of longer chain length. Commercially produced pLL(13) was, however, polymeric and presumably included a significant number of short chain oligomers. Pure oligmers should be synthesised to determine what is the minimum chain length for DNA condensation.

2.10.3. Complexation of DNA using novel cationic polypeptides.

Poly-L-lysine substituted with alanine oligopeptides retained the capability for DNA binding. In this series of molecules, polylysine was derivatised with alanine through the butylamino side chain such that the α-amino group of the alanine residue provided the positive charge. However, for each of the alanine-lysine graft co-polymers the amount of charge required to produce fluorescence quenching was found to be greater than for pLL(214) which binds DNA with 1 to 1 stoichiometry (Tsuboi *et al*, 1966, Olins *et al*, 1967). The observation that fluorescence quenching occurred at a graft co-polymer:DNA charge ratio greater than unity suggests each α-amino group does not interact with the negative charge associated with each nucleotide. This may result from the steric effect produced by displacing the positive charge away from the polymer backbone. Moreover, as the condensation of plasmid-DNA by AK500 was achieved at charge ratios lower than those described for AK100 and AK200 it is apparent that longer chain polymers of this type bind DNA more effectively.

2.10.4. Complexation of DNA using Histone H1.

Early investigations by Olins and Olins, (1971) showed that following complexation of DNA by histone H1, at a sodium chloride concentration of 150 mM, toroid structures were reproducibly formed. These structures were similar to those described for both polylysine (Laemmli, 1975) and transferrin-derivatised polylysine (Wagner *et al*, 1991). However, histone H1 differs from the polymers investigated earlier in this study as at least part of the molecule forms a globular structure in aqueous solution (Bradbury *et al*, 1975b). The retarding of nucleic acids within the gel at a polypeptide:DNA mass ratios of 0.4:1, rather than the formation of aggregates, is consistent with the observations by Johnson *et al*, (1995) who described the preparation of 'slow' complexes. In these systems, histone H1 is

reversibly bound to DNA. However, this type of complexation, is unusual in systems where salt concentrations are greater than 100 mM (Singer and Singer, 1978). That this mechanism was seen under the condition used in this experiment is perhaps a reflection of the conformation of the pRSVlacZ-DNA molecules or the presence of impurities. At mass ratios greater than 0.8:1 the addition of histone H1 to pRSVlacZ also resulted in 'fast' co-operative complexation where DNA was localised in the well of the gel. Clark and Thomas (1986) also describe the formation of complexes by co-operative complexation at high H1:DNA ratios(>1.0% $^{w}/_{w}$) although in these experiments DNA was in a linear conformation.

2.10.5. Complexation of DNA: Comparison of gel mobility shift and ethidium bromide exclusion assays.

As described previously the neutralisation of the anionic charges of DNA by the protonated amino groups of cationic carrier polypeptides produces a change in the electrophoretic mobility of plasmid-DNA. In the gel mobility shift assay this results in a reduction in free DNA and a corresponding increase in complexed DNA which remains at the gel origin. In contrast to the gel mobility shift assay the ethidium bromide exclusion assay is carried out on the bulk sample. In this test, the ability of ethidium bromide to bind DNA in the presence of increasing quantities of polymer is measured spectrofluorometrically. Ethidium bromide has been shown to intercalate into DNA in a manner similar to the acridines (Waring, 1965) and this produces a change in fluorescent yield. The mechanism by which DNA binding of ethidium is inhibited is not fully understood; the cationic polymer may prevent intercalation by blocking the sites of insertion, altering the flexibility or conformation of DNA, or by preventing electrostatic interaction between the ethidium cation and nucleotide phosphates (Olins, 1969).

Comparison of data obtained from the two assays for the complexation of plasmid DNA by poly-L-lysine(214) indicated some inconsistencies. Notably, the apparent

end point for the gel mobility shift assay was achieved with the addition of a mass of polymer which did not produce complete fluorescence quenching in the ethidium exclusion test (total retention of DNA at the gel origin was defined as the end point of the gel mobility shift assay). However, as discussed previously, the ethidium bromide assay showed condensation at charge ratios which were in agreement with generally held theories on DNA collapse. It is therefore possible that this assay may be a more accurate reflection of the subtle changes resulting in DNA condensation.

2.11. Summary.

In summary, complexation of both plasmid and chromosomal DNA by poly-L-lysine was demonstrated using both spectrofluorimetry and gel mobility shift assays. Poly-L-lysine appeared to bind and condense DNA in accordance with the general theories of DNA complexation. The quantity of cationic polypeptide required to complex DNA completely, in terms of both mass and charge, was also determined for alanine-lysine heteropolymers and histone H1. Cationic-polypeptide-DNA complexes formulated using these data were tested in gene delivery studies which are reported in chapter 4.

Chapter 3

Preparation of streptavidin-poly-L-lysine.

Since the first description of receptor mediated gene delivery several methods have been described for conjugating cell surface ligands to DNA-polylysine complexes. The direct coupling of asialoorosomucoid to poly-L-lysine by way of a covalent chemical bond has been achieved using the bifunctional cross-linking reagent N-succinimidyl 3-(2-pyridyldithio)propionate (Wu and Wu, 1987) and the water soluble carbodiimide 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Cristiano *et al*, 1993). The use of SPDP was also the preferred method for covalent linking of transferrin (Wagner *et al*, 1990) and IgG monoclonal antibodies to poly-L-lysine (Rojanasakul *et al*, 1994). An alternative method exploits the specific binding of biotinylated ligands by streptavidin or anti-biotin antibodies (Batra *et al*, 1994). This strategy requires construction of DNA-complexes using poly-L-lysine derivatised with an appropriate biotin binding protein. Targetable vectors can then be formed by addition of biotinylated receptor ligands which are bound to complexes through a high affinity non-covalent interaction. The application of (strept)avidin-biotin technology in forming targeted complexes was prompted by the exceptional affinity ($10^{-15}M^{-1}$) of biotin/avidin binding. Systems of this type allow alteration of the ligand moiety of the construct, which changes the specificity of the vector, without the requirement for several complicated synthesis reactions. The work presented here describes the conjugation of streptavidin to poly-L-lysine.

3.1. Peptide bond formation by carbodiimide reagents.

Carbodiimides are a group of condensing reagents that facilitate the formation of a peptide bond between free amino and carboxyl groups. Practically, this is a one-step

procedure which may be performed at room temperature with subsequent high yields (Sheehan and Hess, 1955). In addition to their use in peptide synthesis, carbodiimide reagents have found various applications in the coupling of small molecule drugs and peptides to carrier polymers and proteins in macromolecular drug delivery systems; e.g. bovine serum albumin linked to α-melanocyte stimulating hormone (McGuire *et al*, 1965) and methotrexate bound to poly-L-lysine (Ryser and Shen, 1978). Directly relevant to this study was the use of the water soluble carbodiimide EDC to prepare hydrazide derivatives of streptavidin for glycoconjugate labelling (Bayer *et al*, 1987). The use of water-soluble carbodiimides for such purposes enables separation of the product from unreacted reagents and reaction by-products by gel filtration and dialysis (Bauminger and Wilchek, 1980).

A general scheme for the linking of poly-L-lysine and streptavidin using a carbodiimide reagent is presented in Figure 3.1. The postulated mechanism for this reaction involves the protonation of the carbodiimide reagent (I) which is subsequently susceptible to attack from the acid anion (II). Rearrangement to an o-acylisurea intermediate (III) produces a species which can react with an amine (IV) to give the desired peptide (V) and a urea (VI) or rearrange to an acylurea (VII). As acyl ureas are mainly produced at elevated temperatures peptide bond formation can be increased by performing the reaction at around 0°C (Bauminger and Wilchek, 1980).

3.2. Physical characteristics of proteins.

Bovine serum albumin (BSA) was used as a model protein in this study as the use of streptavidin in initial conjugation reactions was prohibitively expensive. These globular proteins are similar in size (BSA = 66 kDa; streptavidin = 60 kDa). The isoelectric points of BSA and streptavidin are 4.0 and 5.5 respectively.

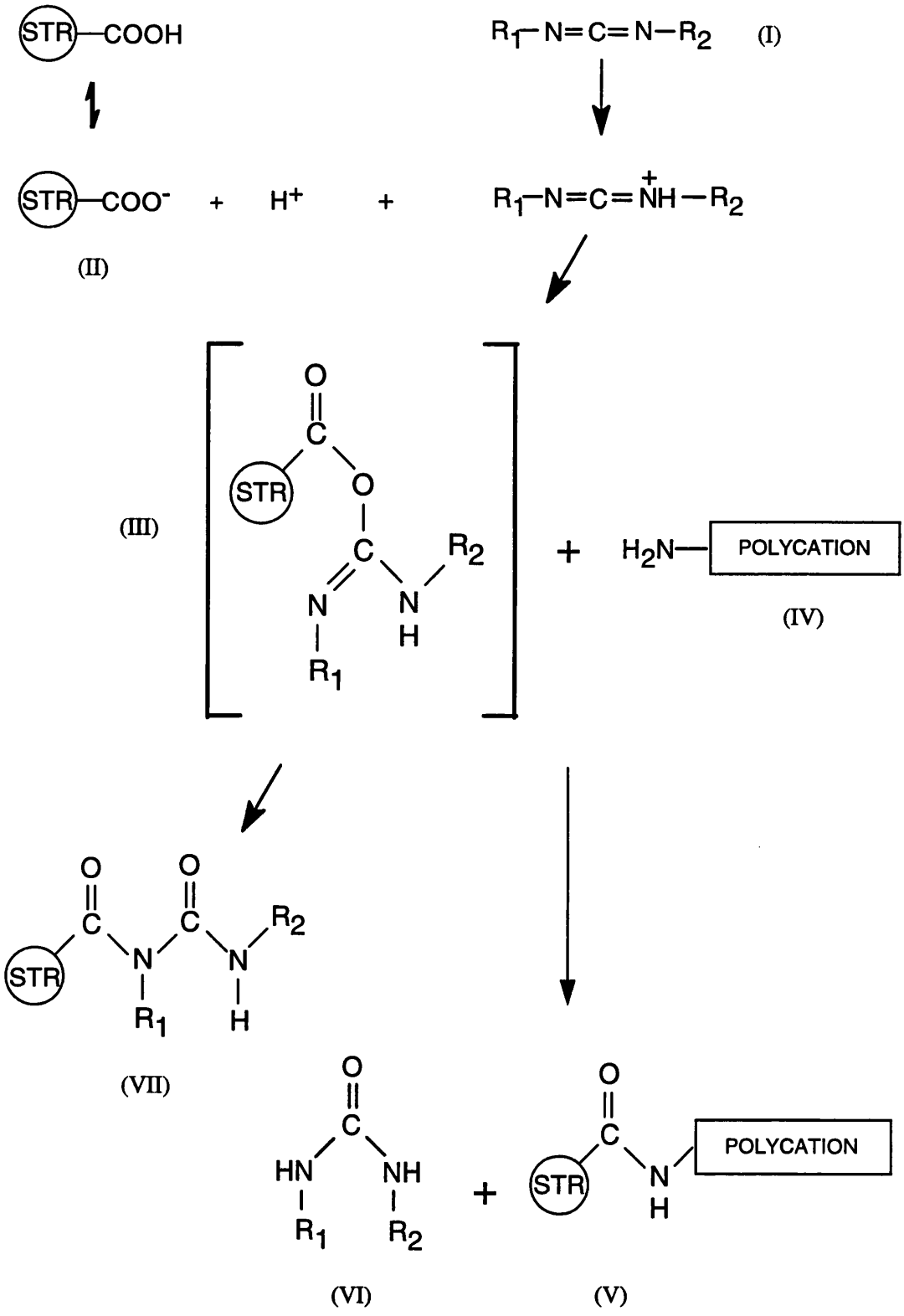


Figure 3.1. Peptide bond formation between streptavidin and poly-L-lysine using carbodiimide reagents. For EDC $R_1=CH_3CH_2$, $R_2=CH_2CH_2CH_2NH^+(CH_3)_2Cl^-$. STR=streptavidin.

3.3. Synthesis of bovine serum albumin-poly-L-lysine conjugates.

Conjugation of bovine serum albumin and poly-L-lysine (219) was performed using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). The optimum quantity of EDC required for efficient coupling was determined by reacting equimolar quantities of BSA and poly-L-lysine hydrobromide using an increasing quantity of EDC. A 0.5 ml aliquot of BSA solution (16 mg/ml) was added to a small glass reaction vial containing 5 mg of poly-L-lysine hydrobromide. After thorough mixing, aliquots of freshly prepared EDC solution (25 mg/ml) were added to the solution and the volume adjusted to 2.0 ml with 50 mM Hepes (pH 7.4). The composition of the reaction mixtures is described in Table 3.1. Following incubation at 4°C for 24 hours the reaction mixtures were separated by ion exchange chromatography (see section 3.4.). Eluate was monitored by flow UV spectrophotometry at 280 nm. Peak areas were then calculated by integration and the proportion of unreacted and conjugated BSA was expressed as a percentage of the BSA standard peak area.

Table 3.1. Composition of reaction mixtures containing BSA and poly-L-lysine.

Reactant	**Stock Solution Concentration**	**Reaction Mixture**					
	-	A	B	C	D	E	F
BSA	16mg/ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml	0.5ml
pLL (219)	-	-	5mg	5mg	5mg	5mg	5mg
EDC	25mg/ml	-	-	0.3ml	0.4ml	0.5ml	0.6ml
Hepes	50mM(pH 7.4)	2.0ml	2.0ml	1.7ml	1.6ml	1.5ml	1.4ml
Molar Ratio (BSA : pLL : EDC)		1:0:0	1:1:0	1:1:357	1:1:477	1:1:596	1:1:715

3.4. Isolation of reaction products using ion exchange chromatography.

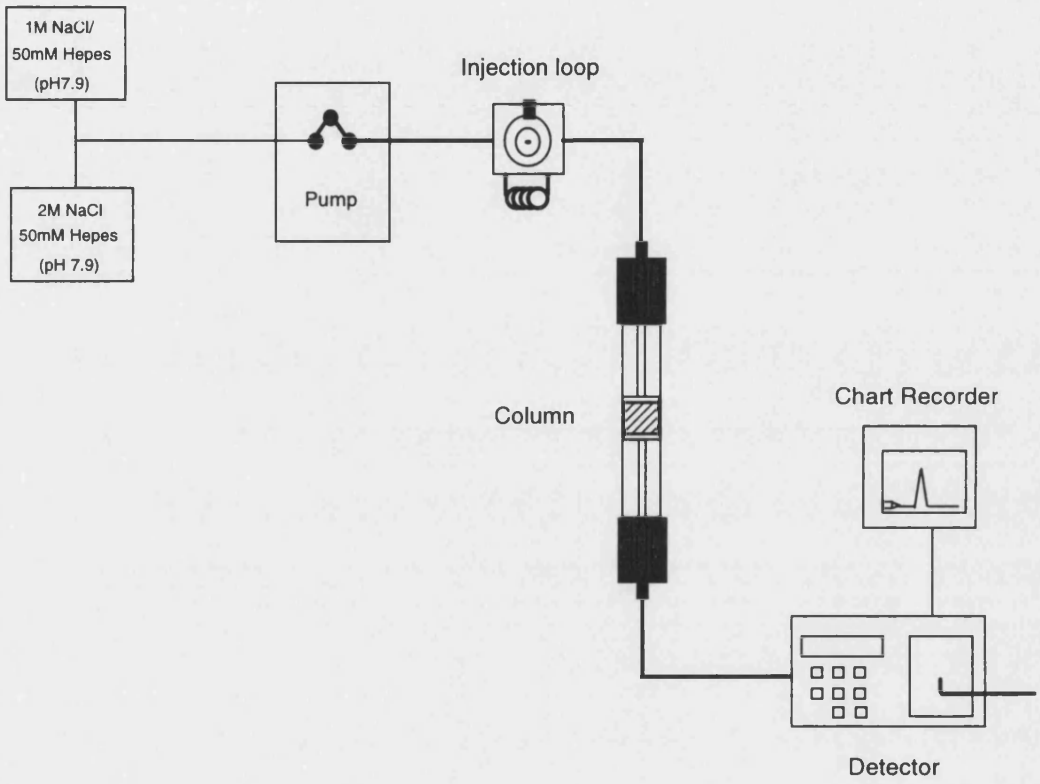
Isolation of proteins modified with poly-L-lysine was carried out by small scale preparative ion-exchange chromatography. A step gradient buffer system was developed based on the continuous gradient described by Wagner *et al*, (1990) for the isolation of transferrin-polylysine. Separation of the reaction products was based on the differential elution of proteins and poly-L-lysine under low and high salt conditions.

SP Sepharose HP ion exchange media (strong cation, Pharmacia) was packed into a 1.0 cm x 20 cm glass column (Pharmacia) to give a settled volume of 2.0 cm^3. Initially, buffer A was used as the eluent. The reaction mixture was then applied to the column through a syringe loading sample injector connected to a 2.0 ml calibrated loop. The eluate was monitored by flow UV spectrophotometry at 280 nm. A schematic diagram of the chromatography system is provided in Figure 3.2. The first peak was eluted in buffer A [50 mM Hepes/1M NaCl (pH 7.9)] and collected in a 4.0 ml glass vial. A second peak was then eluted using buffer B [50 mM Hepes/2M NaCl (pH 7.9)].

3.5. Synthesis of streptavidin-poly-L-lysine conjugates.

Recombinant streptavidin (Sigma) was coupled to poly-L-lysine (M_r = 28000) according to the general method described in section 3.3. Sixty-seven nanomoles of streptavidin (4.0 mg) were reacted with an equimolar quantity of poly-L-lysine (3.1 mg; free base) in 1380 µl of 20 mM Hepes (pH 7.4) using a 600 fold molar excess of EDC. After incubation for 24 hours at 4°C, the reaction was terminated by fractionation on an ion exchange column (see section 3.4.). Fractions were collected in sterile 4 ml glass vials. Poly-L-lysine-streptavidin adducts were prepared under clean laboratory conditions to minimise microbiological contamination.

Figure 3.2. Schematic diagram of ion exchange chromatography system.



Mobile phase:	Buffer A: 50mM Hepes/1M NaCl (pH 7.9)
	Buffer B: 50mM Hepes/2M NaCl (pH 7.9)
	Filtered (0.2 μm), degassed
Pump:	Gilson Minipuls 2, 1.0 ml/min flow rate
Injector	Rheodyne 7132
Column	C 10/20 (Pharmacia)
Detector	Gilson Model 116
Chart recorder	BBC Servogor 120
Intergrator	Milton Roy CI-4100

3.6. Dialysis of fractions isolated by ion exchange chromatography.

Following separation by cationic exchange chromatography, the fractions isolated in 1M NaCl/50 mM Hepes (pH 7.9) and 2M NaCl/50mM Hepes (pH 7.9) were placed in dialysis tubing (10 mm flat width) with an exclusion limit of 12,000 to 14,000 daltons (Spectrum Medical Industries, Los Angeles, CA, USA). Plastic closures were used to secure the ends of the tubes. Dialysis was carried out at 4°C for 24 hours against 1000 ml of 150 mM NaCl/50 mM Hepes (pH 7.4) in a conical flask with constant stirring. The buffer was changed once during dialysis. Prior to use, the dialysis tubing was cut to an appropriate length and boiled for 10 minutes in a 2 mM solution of EDTA then washed thoroughly in double distilled water. This washing procedure was repeated before the tubing was placed in a 100 ml Duran® bottle, submersed in double distilled water and autoclaved for 15 minutes at 121°C.

3.7. Analysis of conjugate composition.

Composition of conjugates and calculation of total yield was established by quantitative assay of samples following dialysis. Standard curves are presented in appendix 3.

3.7.1. Quantification of streptavidin content of conjugates.

Streptavidin content of conjugates was determined by UV spectrophotometry at 280 nm. Poly-L-lysine does not absorb UV radiation at this wavelength. Values were corrected by subtracting the corresponding UV absorbance of HBS at 280 nm. A standard curve was prepared using streptavidin of the specific batch used in the synthetic reaction. A representative curve is presented in section A3.1.

3.7.2. Ninhydrin assay for primary amines.

Poly-L-lysine content of fractions was determined spectrophotometrically according to the ninhydrin method modified from Blackburn, (1968). Samples (75-300 µl) were transferred into 16 x 150 mm test tubes and made up to 1.0 ml total volume with 50 mM Hepes/150mM sodium chloride (pH 7.4). Then, 1.0 ml of ninhydrin reagent solution (ninhydrin $20g/l^{-1}$, hydrinantin $3g/l^{-1}$, lithium acetate 1M, dimethylsulphoxide 75% $^{w}/_{v}$) was added and the samples vortexed. The tubes were capped and placed in a heating block (Techne DB-2A) pre-adjusted to 100°C. After exactly ten minutes, the tubes were removed and placed on ice. A 1.0 ml aliquot was removed and diluted to 5.0 ml with water. The absorbance of each solution was measured against the reaction blank, prepared from buffer, at 570 nm using a Milton Roy 601 spectrophotometer. A standard curve was prepared using poly-L-lysine (see section A3.2.). Triplicate assays were conducted for each sample and the mean value used for calculation of polymer concentration. At the concentrations present in the conjugate samples, streptavidin produced negligible responses.

3.8. Electrophoresis of fractions isolated by ion exchange chromatography to confirm their identity.

Following ion exchange chromatography, purified fractions were analysed by SDS/PAGE, PAGE and affinity blotting to determine their constituents. Electrophoresis was carried out in the Mini-Protean® system (Bio-Rad) using de-naturing and native 15% polyacrylamide gels prepared according to the formulations in Table 3.2.

Sample preparation for SDS-PAGE was by boiling one volume of eluate with 0.2 volumes of sample buffer for 10 minutes. Electrophoresis was carried out at 200V until the tracking dye, or appropriate prestained marker, reached the bottom of the

gel. Native gels were prepared for PAGE in a similar manner, although sodium dodecyl sulphate was removed from both the gel formulation and sample buffer. Samples prepared for PAGE were not boiled.

3.8.1. Visualisation of proteins.

After electrophoresis, gels were removed from the glass plates and proteins were either stained directly or transferred to nitrocellulose for subsequent reaction with biotin-DAPA-peroxidase (Sigma).

Directly stained gels were soaked, with gentle agitation in 0.1 % $^{w}/_{v}$ Coomassie Brilliant Blue R-250 in 50 % methanol/10% acetic acid for 60 minutes. Gels were then destained in 10% methanol/20 % acetic acid until the background stain was removed. Gels were dried onto filter paper using a Model 583 slab dryer (Bio-Rad). A drying temperature of 70°C and a 60 minute cycle was used for mini-gels.

3.8.2. Affinity blotting of biotin binding proteins.

Following separation by electrophoresis, proteins were transferred onto nitrocellulose membranes (0.45 µm pore size, Bio-Rad) according to the western blotting method modified from Towbin *et al*, (1979). Transfer was carried out using a Mini-Trans blot cell (Bio-Rad), (25 mM Tris, 192 mM glycine and 20 % $^{v}/_{v}$ methanol, pH 8.3), at 100V for one hour.

Transferred streptavidin was detected using a biotin-DAPA-peroxidase conjugate, prepared from Type VI horseradish peroxidase (Sigma). Blots were first washed for one hour with gentle agitation in TBS containing 0.15% $^{v}/_{v}$ Tween 20. This blocked unbound sites on the nitrocellulose membrane. Following this blots were then rinsed thoroughly in TBS and soaked, with gentle agitation, in TBS/0.15% Tween 20 containing 2 µg/ml biotin-DAPA-Peroxidase for 3 hours at room temperature. This

solution was removed, and the blots washed again with TBS. Blots were visualised with freshly prepared developing solutions. Immediately before developing the blot, 0.5 ml of 3% $^{w}/_{v}$ 4-chloro-1-napthol in methanol and 50 µl of 6% $^{w}/_{v}$ hydrogen peroxide was added to 50 ml of 10 mM Tris (pH 7.4). The blot was exposed to the developing solution (4-chloro-1-napthol 30 µg/ml, hydrogen peroxide 60 µg/ml, 10 mM Tris, pH 7.4) for 30 minutes. This solution was warmed slightly to speed the reaction. Colour development was allowed to proceed for 30 minutes before the reaction was stopped, to avoid high background, by washing with distilled water.

Table 3.2. Composition of running gel and stacking gel for SDS-PAGE.

Constituent	Running Gel (15%)	Stacking Gel (5%)	Sample buffer
Stock 1	10.0 ml	1.7 ml	-
SDS 10% $^{w}/_{v}$	0.2 ml	0.1 ml	5 ml
1.5M Tris pH 8.8	5.0 ml	-	-
0.5M Tris pH 6.8	-	1.25 ml	2.5 ml
Distilled Water	4.6 ml	6.8 ml	5 ml
AMPS 10% $^{w}/_{v}$	0.2 ml	0.1 ml	-
TEMED	10 µl	10 µl	-
2-mercaptoethanol	-	-	0.25 ml
Bromophenol Blue 5% $^{w}/_{v}$	-	-	0.2ml
Glycerol	-	-	2.5 ml

Stock 1: 30% $^{w}/_{v}$ acrylamide and 0.8 % $^{w}/_{v}$ N,N'-methylene-bis-acrylamide (Bis) Final acrylamide concentration = 15% in the running gel and 5% in the stacking gel.

3.9. Formation of streptavidin-poly-L-lysine-DNA complexes.

Complexes of various streptavidin-polylysine:DNA ratios were formed by adding a solution containing 0-4.2 μg of the polypeptide (77 μg/ml; calculated as free polylysine) to a solution containing 6 μg DNA (24 μg/ml). The complexation reaction was otherwise as described in section 2.4.

3.9.1. Analysis of DNA complexation.

Throughout this study two batches of streptavidin-polylysine conjugates (Bn 001 and Bn 002) were prepared using the synthetic method described in section 3.5. The composition of conjugates was analysed as described in section 3.7. In addition, to ensure reproducibility, the ability of differing batches of Str-pLL to complex DNA was compared by spectrofluorimetry (see section 2.5.1.).

RESULTS.

3.10. The effect of EDC concentration on the conjugation of BSA to polylysine.

Data illustrating the influence of EDC concentration on the conjugation of BSA and poly-L-lysine are described in Table 3.3. Varying the relative concentration of EDC (BSA:pLL:X) gave an increase in BSA eluted in buffer B, from 9.6 % (at 1:1:0) to 83.8 % (at 1:1:715). This was accompanied by a decrease in BSA eluted in buffer A. The curves illustrating the relationship between conjugation of BSA and EDC concentration were linear at lower concentrations, with deviation from linearity at higher concentrations (Fig. 3.3). Mixing of BSA and poly-L-lysine in the absence of the coupling reagent resulted in the elution of 9.6% of BSA in buffer B suggesting a non-covalent interaction between the reactants. The mean yield of BSA eluted in

buffer A and buffer B was 96.4% of the standard peak area, indicating a minimal loss of protein during product isolation.

Table 3.3. Conjugation of BSA and poly-L-lysine (219). Equimolar quantities of BSA and polylysine were coupled using increasing quantities of EDC in 50 mM Hepes (pH 7.4). Reaction mixtures were separated by ion exchange chromatography as described in section 3.4. The quantity of BSA eluted in each buffer is expressed as a percentage of the standard peak area. The minimum and maximum data points are also shown.

Molar Ratio BSA:pLL:EDC	Percentage of BSA input eluted in Buffer A Mean (Min ; Max)	Percentage of BSA input eluted in Buffer B Mean (Min ; Max)	Percentage of BSA input eluted in Buffer A and B Mean (Min ; Max)	n
1:1:0	89.1 (83.7 ; 96.4)	9.6 (4.9 ; 17.0)	98.8 (94.3 ; 100.7)	3
1:1:238	54.2 (52.9 ; 55.5)	37.9 (37.4 ; 38.4)	92.1 (90.3 ; 93.9)	2
1:1:357	39.3 (35.4 ; 44.5)	60.2 (58.1 ; 62.2)	99.5 (93.5 ; 106.7)	3
1:1:477	24.8 (24.2 ; 25.3)	71.6 (68.0 ; 75.7)	96.4 (93.3 ; 96.0)	3
1:1:596	17.5 (14.9 ; 23.3)	76.8 (71.8 ; 81.2)	94.3 (86.7 ; 104.5)	3
1:1:715	13.6 (12.1 ; 16.2)	83.8 (80.3 ; 89.3)	97.4 (92.4 ; 101.7)	3

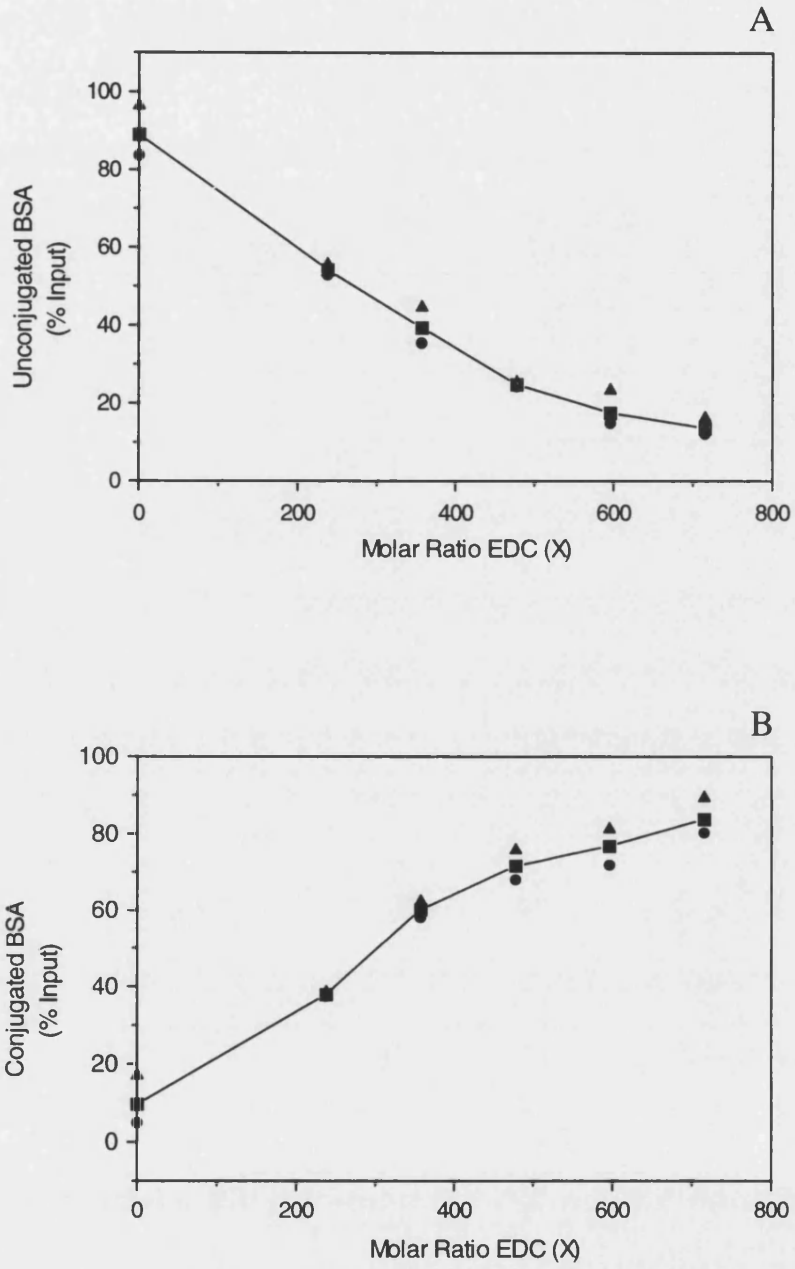


Figure 3.3. Conjugation of BSA and poly-L-lysine (219). BSA and pLL were coupled using increasing quantities of EDC (Molar ratio BSA:pLL:EDC=1:1:X). The reaction mixtures were separated using ion exchange chromatography. The proportion of BSA eluted in buffer A (Panel A) and buffer B (Panel B) was calculated as a percentage of the BSA standard peak area. The mean (■), minimum (●) and maximum (▲) values are shown for each EDC concentration.

3.11. Isolation of streptavidin-poly-L-lysine conjugates by cation exchange chromatography.

Streptavidin-poly-L-lysine was separated from the reaction mixture using the chromatography system described in section 3.4. A representative chromatogram for the separation is shown in Figure 3.4. Peak A was eluted in 1M NaCl/50 mM Hepes (pH 7.9). A second peak (B), which was believed to contain the product, was then eluted when the buffer was changed to 2M NaCl/50 mM Hepes (pH 7.9).

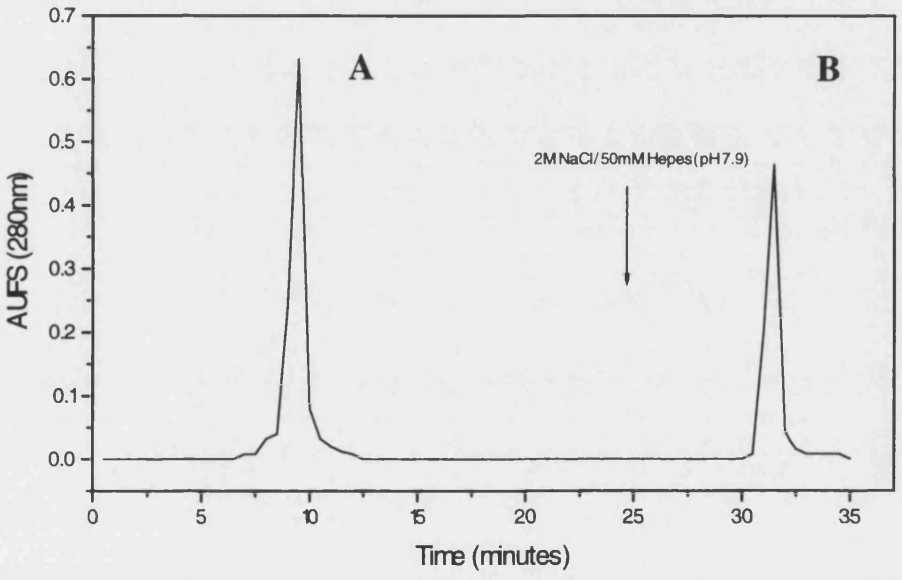


Figure 3.4. Purification of streptavidin-poly-L-lysine(219) conjugates. Separation of reaction mixtures was by cation-exchange chromatography.

3.11.1. Identification of peak constituents.

Following separation peak fractions were analysed by qualitative ninhydrin assay, gel electrophoresis and affinity blotting to identify the fraction containing the streptavidin modified polylysine conjugate. Initial qualitative ninhydrin tests indicated the presence of polyamines in fraction B; a positive colour reaction was observed after 5 minutes. Fraction A was negative for primary amines. In each case the volume of the samples analysed were adjusted to compensate for variation in the streptavidin content. Fraction's A and B both contained streptavidin as indicated by A_{280} values.

To examine the coupling of streptavidin and poly-L-lysine, ion exchange fractions were analysed by SDS-PAGE. The electrophoretic pattern of proteins eluted in buffer A and B were identical, showing a major doublet band at a mass less than 18.5 kDa and a minor band at 32 kDa (Fig. 3.5.). A positive reaction to biotin-DAPA-peroxidase was also exhibited by these bands on affinity blotting. The multiple bands are likely result from the method used to isolate the streptavidin (Bayer *et al*, 1986). To determine if the apparent migration pattern indicated chemical instability of the conjugate, or resulted from the disruption of protein secondary structure, samples were also analysed by PAGE. However, as the undenatutred form of this protein stained poorly with coomassie blue reagent, detection was carried out using biotin-conjugated-peroxidase after electro-transfer onto nitrocellulose membranes. A representative result obtained for the streptavidin-polylysine conjugate is shown in Figure 3.6. In this non-denaturing system, streptavidin migrated as a single band and gave a specific reaction to biotin-DAPA-peroxidase. Streptavidin was detected in fraction A but was not seen in fraction B. This result indicates the absence of free streptavidin in the reaction product. The positively charged streptavidin-polylysine molecule is not transferred to the nitrocellulose membrane.

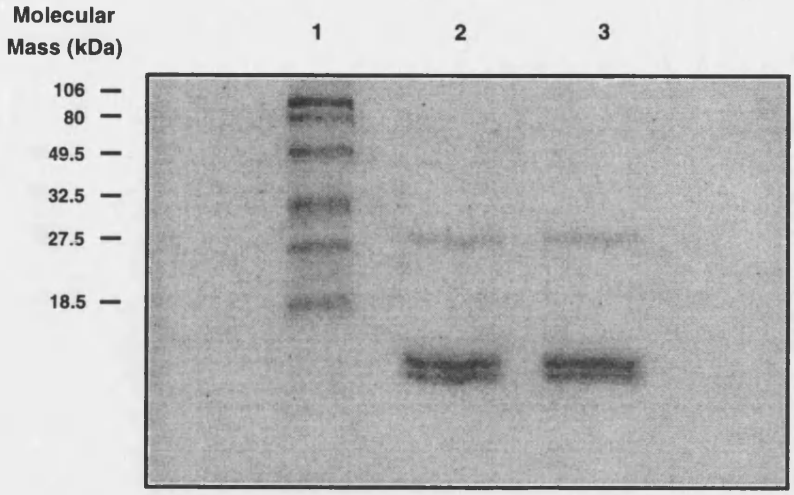


Figure 3.5. SDS-PAGE analysis of ion-exchange fractions. Protein was detected by coomassie blue staining. Lane 1, Molecular weight standards; Lane 2, Fraction A; Lane 3, Fraction B.

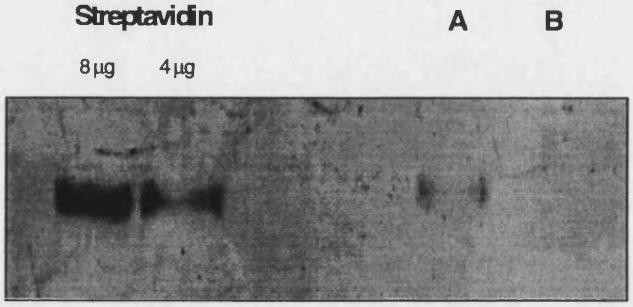


Figure 3.6. Affinity blot analysis of fractions purified by ion-exchange chromatography. Aliquots of fractions A and B containing streptavidin (1.2 µg) were analysed using PAGE and electro-eluted onto nitrocellulose membranes as described in section 3.8.2. Streptavidin was detected using biotin-DAPA-peroxidase. Control lanes of unreacted streptavidin were also included.

3.11.2. Quantitative analysis of conjugate composition.

During this study two batches of conjugates containing poly-L-lysine modified with streptavidin were prepared. Results from quantitative analysis of these batches following dialysis is shown in Table 3.4. The yield of conjugate and the level of streptavidin substitution of polylysine was similar for each batch. These data were used in calculating the polycation concentration for complexation studies

Table 3.4. Quantitative analysis of conjugate composition.

	Input		Product		
Batch	pLL (nanomoles)	Streptavidin (nanomoles)	pLL nanomoles (% Input)	Streptavidin nanomoles (% Input)	Molar Ratio pLL:Str
001	67	68	39 (57.4%)	17.3 (25.8 %)	1:0.44
002	67	68	33 (47.8%)	14.0 (20.9 %)	1:0.43

3.12. Complexation of DNA using streptavidin modified polylysine.

Figure 3.7. shows the mobility shift assay performed on complexes formed between streptavidin modified polylysine and pRSVlacZ-DNA. Movement of plasmid-DNA was initially retarded at a DNA-to-polypeptide mass ratio of 0.14:1 with the amount of unbound plasmid decreasing with increasing polymer concentration. Complete association of plasmid DNA into complexes was seen at the mass ratio of 0.37:1. Further analysis of DNA condensation was carried out using the ethidium bromide exclusion assay described in section 2.5.1. Following appropriate dilution's the exclusion of ethidium bromide produced by each batch of conjugate was similar; a reduction of fluorescence intensity to background levels being achieved at a polypeptide-to-DNA mass ratio of 0.43:1 which corresponds to a charge ratio (+/-) of 1.1. In the calculation of charge ratios, corrections were not made for the streptavidin, which would be negatively charged at pH 7.4, as it was considered that only positively charged poly-L-lysine molecules would be involved with DNA condensation.

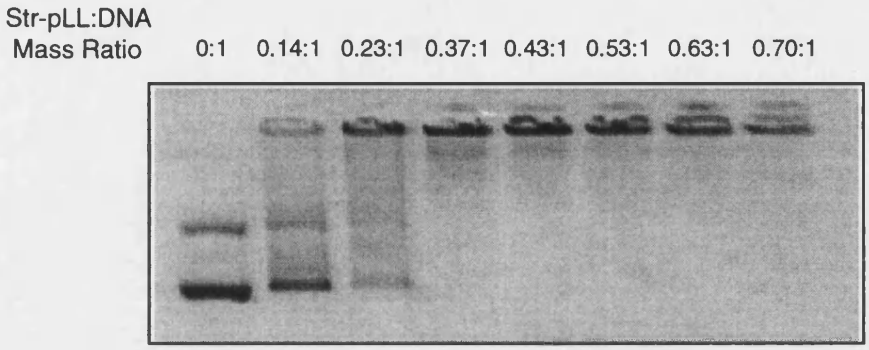


Figure 3.7. Analysis of pRSVlacZ complexed with increasing amounts of streptavidin-polylysine. Complexes were prepared as described in section 3.8 and resolved through a 1% agarose gel. 0:1 represents pRSVlacZ only; bands represent from bottom to top of gel, plasmid DNA in supercoiled and open circle conformations.

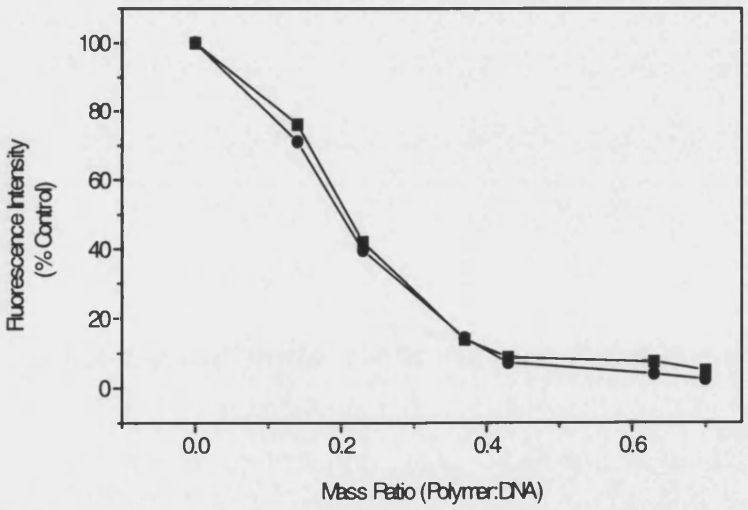


Figure 3.8. Effect of formulation in terms of polymer:DNA mass ratio on fluorescence intensity for the quantitative assessment of batch variation. After appropriate dilution of stock solutions complexes were formed between pRSVlacZ and batch 001 (■) and batch 002 (●) of streptavidin-poly-L-lysine adducts.

3.13. Discussion.

3.13.1. Conjugation of bovine serum albumin and streptavidin to poly-L-lysine using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

The conjugation of the 60 kDa biotin binding protein streptavidin to a cationic polypeptide (polylysine) was achieved using a simple one step procedure employing EDC to create an amide bond between carboxylic acid residues on the protein and an amine group on the polymer. This reaction has been used previously for the coupling of asialorosomucoid to polylysine with separation of the products and reactants achieved by FPLC (Cristiano *et al*, 1993). In this study cation-exchange chromatography followed by dialysis was used to isolate the conjugates.

Bovine serum albumin (BSA) appeared to provide a good model compound for initial conjugation reactions as a result of similarities in size and charge to streptavidin. Therefore, the optimal conditions for the preparation of streptavidin modified polylysine were determined from experiments with BSA. The preparation of a streptavidin modified polylysine conjugate was first documented by Wagner *et al*, (1992a). However, this strategy requires the modification of polylysine to give a reactive group which is then coupled with 3-(2-pyridyldithio)propionate-modified streptavidin. The protein and polycation are linked through a reducible disulphide bridge. In contrast to the above method, using EDC to couple streptavidin and polylysine would yield a stable amide bond between the reactants and does not require the purification of intermediates.

The pH chosen for the reaction of protein and polypeptide was a compromise since the EDC link to acid residues is promoted by hydrogen ions whilst lysine residues are more reactive at elevated pH where amine groups are predominantly unprotonated. Reactions were, therefore, carried out at pH 7.4. Experiments to

determine the optimum quantity of EDC for coupling of BSA to poly-L-lysine gave the expected results; conjugation efficiency was directly related to the concentration of EDC in the reaction mixture (BSA and pLL constant). An apparent complexation of poly-L-lysine and BSA was also observed without EDC (9.6 % of BSA input eluted in buffer B). This was thought to result from an ionic interaction between the polypeptides; the possibility of which is indicated by the relative pI values for BSA (4.0) and poly-L-lysine (11.5). The curvilinear relationship between conjugation efficiency and EDC concentration suggested coupling was limited by level of reactants. However, as the structure of poly-L-lysine provides a large molar excess of reactive sites then the plateau in conjugation efficiency was therefore more likely to be related to formation of active intermediates with carboxyl groups of BSA. It was unlikely that all these residues will be accessible due to the globular nature of the protein.

Using this data a 600 fold molar excess of EDC was selected for the reaction of streptavidin and polylysine. In preliminary experiments these reaction conditions had yielded a high level of conjugation between BSA and polylysine with 76% of BSA conjugated to poly-L-lysine. However, as these conditions resulted in only 47% of applied streptavidin being coupled to poly-L-lysine then the ideality of BSA as a model protein was questioned. The analysis of protein sequence data for BSA and streptavidin (Seqnet, SERC, Daresbury, UK.) showed the former to possess a greater number of acidic residues available for linking to polylysine (99 versus 52). If a similar proportion of these residues are accessible in each protein then it is likely that the number of reactive sites on streptavidin was limiting. The yield of streptavidin in the modified conjugate was calculated from the weight of starting reactants and at 25.8% of input for Batch 001 was significantly lower than that described by Wagner *et al*, (1992a) for a similar conjugate (56% of input) prepared using N-succinimidyl 3-(2-pyridyldithio)propionate.

3.13.2. Separation of reaction products by ion exchange chromatography.

Separation of the reactants and products was successfully achieved using cation exchange chromatography. The excess of uncoupled streptavidin was eluted first using 1M NaCl/50mM Hepes (pH 7.9); streptavidin-poly-L-lysine adducts were retained on the column due to their high positive charge density and were only eluted on changing the buffer to 2M NaCl/50mM Hepes (pH 7.9). This second peak gave a positive colour reaction for amines with ninhydrin.

Initial attempts to validate the isolation process by analysis of fractions by SDS-PAGE failed to detect any difference in the rate of migration of protein eluted in buffers A or B. This raised the possibility that following isolation the conjugate was unstable resulting in separation of streptavidin from the cationic DNA binding moiety. However, non-covalent interactions between protein sub-units are known to be sensitive to denaturing conditions. Indeed, Sheldon *et al*, (1992) have described the dissociation of sub-units from a monoclonal antibody-streptavidin conjugate during separation by SDS-PAGE. In this system, protein sub-units subsequently migrated at an M_r of 15 kDa. SDS-PAGE was unsuitable for analysis of coupling as the detergent component off the system promoted dissociation of the non-covalently bound protein sub-units. Conjugate stability was, therefore, confirmed by analysis of samples on a native polyacrylamide gel and transfer of protein to a nitrocellulose membrane. In this case a reactive streptavidin band was detected in the sample from the first peak but was not present in the product fraction. The streptavidin-polylysine adducts, which have a high positive charge density, are unable to enter the gel. These results are consistent with the isolation of the product under high salt conditions [2M NaCl/50 mM Hepes (pH 7.9)] with unreacted streptavidin eluted in 1M NaCl/50 mM Hepes (pH 7.9).

3.13.3. DNA complexation by streptavidin-poly-L-lysine.

As expected from the work described by Wagner *et al*, (1991), poly-L-lysine retained the ability to complex and condense DNA following derivatisation with a large protein molecule. Gel mobility shift assays indicated a decrease in free plasmid DNA as a function of increasing polypeptide concentration. Indeed, comparison of the fluorescence quenching profiles produced by the native and derivatised cationic polymer showed condensation was induced at the same polypeptide-to-DNA mass ratio (calculated as polylysine). Furthermore, the streptavidin modified polylysine adducts from each batch of conjugate appeared to complex DNA in a similar manner. It was therefore likely that complexes would behave similarly in the *in vitro* transfection experiments.

3.14. Summary.

The carbodiimide method of conjugation gave a streptavidin-modified polylysine conjugate that retained the ability to bind and condense DNA. This conjugation method proved to be reproducible between batches. However, the reaction was inefficient in comparison to the method described by Wagner *et al*, (1992a) although it conferred two distinct advantages; the procedure was less complex requiring a single chromatographic separation and the linkage formed was non-reducible making it more applicable for use in biological systems. Ion exchange chromatography was suitable for isolation of the reaction product, which was free from unconjugated streptavidin. This product was used for receptor-mediated gene delivery studies and to examine the formulation requirements for this process.

Chapter 4

Transfection of B16 murine melanoma cells.

Prior to the construction of poly-L-lysine-DNA-ligand complexes for gene targeting studies it was necessary to determine the ability of the carrier system to transfer DNA *in vitro*. The studies presented here aim to determine the factors which influence gene transfer using streptavidin-poly-L-lysine-DNA formulations; changes in complex composition, dose, dosing schedule, and external factors such as the time course of expression, presence of serum and conjugate stability are examined. To determine the optimal conditions for delivery using polycation systems the addition of agents that affect the survival of transfected DNA in the intracellular environment was also assessed. Finally, histone H1 was tested as an alternative carrier protein.

4.1. Cell Culture.

Minimum Essential Medium with Earle's salts, RPMI-1640 medium, MEM vitamin solution, MEM non-essential amino acids, and penicillin/streptomycin were obtained from Gibco (Paisley). Fetal calf serum was purchased from ICN/Flow (High Wycombe). Water was freshly double distilled, deionised and autoclaved at 121°C for 15 minutes. Tissue culture flasks were from Falcon and 6 well plates from Nunc.

4.1.1. Cell line maintenance and subculture.

B16 mouse melanoma cells were donated by L.R. Kelland (Institute of Cancer Research, Sutton). Cells were maintained in a LEEC anhydric incubator at 37°C in a humidified atmosphere under standard conditions of 95% air/5% CO_2. In order to maintain a pH of 7.2-7.4, the medium was routinely changed the day before the cells

reached confluence. Subculture was undertaken with confluent cells by washing the cell monolayer twice with PBS and then incubating with a sufficient volume of 0.02% $^{w}/_{v}$ EDTA/PBS for 10 minutes. Detached cells were then diluted to 10 ml with culture medium and each 175 cm^2 flask inoculated with 2 x 10^6 cells. For transfection experiments, cells were grown in MEM medium containing 10% fetal calf serum (FCS), 2 mM L-glutamine, 1% MEM non-essential amino acids, 50 IU/ml penicillin, and 50 mg/ml streptomycin. Cells used in receptor binding experiments were grown in RPMI-1640 medium (see section 6.4.). The same routine was used to maintain cultures for this assay.

4.2. Analysis of β-galactosidase expression.

Gene transfer efficiency was determined *in vitro* using plasmid DNA which encoded the *E.coli lacZ* gene for β-galactosidase under the control of the Rous sarcoma virus long terminal repeat sequence. Use of reporter gene technology to monitor delivery efficiency obviated the difficult and time consuming need for direct assays of mRNA. The use of the *lacZ* gene allowed several assays to be performed. Total enzyme activity was determined in cell extracts using a spectrophotometric method based on the conversion of o-nitrophenol-β-D-galactopyranoside (ONPG) to the chromophore o-nitrophenol (Miller, 1972). This assay was performed on cell extracts and standardised by determining the total soluble protein content of each well. Distribution of expression within cell populations was determined either histochemically, using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) using the method described by MacGregor *et al,* (1991), or quantitatively by flow cytometry (Nolan *et al,* 1988).

4.2.1. Preparation of cell extracts.

The medium was removed from the culture and the cells washed three times with 5 ml of PBS. A 1.0 ml aliquot of PBS was then added to the plate and the cells

removed by gentle scraping with a cell scraper and transferred to a 1.5 ml microcentrifuge tube. When multiple extracts were prepared, the cell scraper was washed in 70% ethanol, rinsed in water and blotted dry between samples. Cells were collected by centrifugation and resuspended in 250 µl of 0.1 M sodium phosphate buffer (pH 7.4). The cells were then resuspended and subjected to three freeze thaw cycles (freeze in a dry ice-ethanol bath and thaw at 37°C). The sample was centrifuged for 5 minutes at 5,000 rpm and the supernatant carefully removed to a clean microcentrifuge tube. Extracts were analysed immediately.

4.2.2. Quantitative assay of β-galactosidase activity in cell extracts.

Cell extract supernatant (5-100 µl) was adjusted to 300 µl total volume using double distilled water. An equal volume of 2X ONPG assay buffer (1.35 mg/ml o-nitrophenylgalactopyranoside, 2 mM $MgCl_2$, 100 mM 2-mercaptoethanol, 200 mM sodium phosphate, pH 7.3) was then added to the cell extract solution, mixed, and incubated in a water bath at 37°C. Prior to mixing, all solutions were pre-equilibrated at 37°C for 10 minutes. A blank reaction, which contained no cell extract, was also prepared. After exactly 30 minutes the reaction was terminated by the addition of 1.0 ml 1M sodium carbonate. Reaction mixtures were then transferred to disposable microcuvettes and the absorbance of the solution measured at 420 nm. A standard curve for this assay was prepared using purified *E.coli* β-galactosidase (Promega) and is presented in section A3.3. Total enzyme activity in cell extracts was expressed as milliunits or converted to relative activity by dividing the amount of β-galactosidase (milliunits) recovered per well by the amount of protein (milligrams) remaining in each well at the end of the period allowed for gene expression. In this thesis, total activity data is presented when the toxicity of test conditions reduced cell viability.

4.2.3. Quantification of soluble protein in cell extracts.

The soluble protein content of cell extracts was determined using DC assay reagent (Bio-Rad). This assay is based on the method of Lowry *et al,* (1951). For assaying protein, 25-100 µl of cell extract supernatant was added to 100 mM sodium phosphate buffer, pH 7.4 to give 100 µl total volume. The assay was then performed in accordance with manufacturer's instructions. Absorbances were measured at 750 nm. Standard curves were prepared using bovine serum albumin. A representative curve is presented in section A3.4.

4.2.4. In situ cytochemical staining for β-galactosidase activity.

B16 melanoma cells transfected with the β-galactosidase reporter gene were detected, *in situ*, using a method modified from MacGregor *et al,* (1991). Forty eight hours after transfection the growth medium was removed from the adherent cell monolayers. The cells were then washed twice with PBS and over-layered with fixative solution (1% glutaraldehyde, 1 mM magnesium chloride, 100 mM sodium phosphate) for five minutes at 4°C. The fixative solution was removed by two washes with PBS. A 2% $^{w}/_{v}$ solution of X-gal in dimethylformamide was diluted to 2 mg/ml in 3.3 mM potassium ferrocyanide, 3.3 mM potassium ferricyanide, 1 mM magnesium chloride, 150 mM sodium chloride and incubated with the cells for four hours in a humidified atmosphere at 37°C. All solutions were prepared in glass. After staining, cells were washed three times with PBS and then examined under a light microscope. Cells expressing the *lacZ* gene hydrolyse the chromogenic substrate X-gal to give the dye bromochloroindole. A positive reaction is seen as a blue colour against an unstained background.

4.2.5. Analysis of β-galactosidase expression by flow cytometry.

The proportion of the total cell population which expressed β-galactosidase following transfection with pRSVlacZ was determined by FACS analysis using the

method described by Nolan *et al,* (1988). A 200 mM stock solution of the fluorescent probe fluorescein-di-β-D-galactopyranoside (FDG) was prepared by dissolving 1.5 mg of the pale yellow solid in 11.4 µl of DMSO:water (1:1 v/v). Since the mixing of DMSO and water is exothermic it was necessary to cool the mixture before dissolving FDG. The 2 mM working solution of FDG was prepared by adding 11 µl of 200 mM FDG to 1090 µl water and was stored in 300 µl aliquots at -20°C. Staining medium (SM:10 mM Hepes, 4% v/v FCS: pH 7.4) was prepared immediately before use using 2x SM salts and fetal calf serum.

4.2.5.1. Cell preparation and FDG staining.

Medium was removed from the confluent culture and the cells were washed twice with 2.0 ml PBS. Adherent cells were removed using 0.02% w/w EDTA/PBS, transferred to a 1.5 ml microcentrifuge tube and collected by centrifugation at 10,000 rpm for 10 minutes. The cell pellet was resuspended in 250 µl SM and a 50 µl aliquot of the cell suspension transferred to a 4 ml FACS tube (Falcon). The tube containing the cell suspension was then placed in a water bath for 10 minutes at 37°C before 50 µl of prewarmed 2 mM FDG was added. The suspension was mixed rapidly by vortexing and returned to the 37°C water bath for exactly 60 seconds. FDG is passively taken up into the cells during this brief hypotonic shock. Loading was stopped by the addition of 1900 µl of ice cold isotonic SM and the tube placed on ice for 60 minutes to allow FDG hydrolysis. SM was transferred using automatic pipette tips chilled at -20°C. Immediately prior to FACS analysis 10 µl propidium iodide (20 mg/ml) was added to each sample. Analysis was performed with live gating as described in section 4.10. Compensation was made for cell autofluorescence by incubating untransfected cells with 50 µl of water during the loading step. Endogenous β-galactosidase activity of B16 cells was measured by incubating untransfected cells with FDG.

4.3. Transfection of B16 melanoma cells.

For gene transfer experiments, cells were removed from adherent cultures using the method described in section 4.1.1. and plated at a density of 1 x 10^5 cells per well of a six well plate (35 mm diameter). The cells were then allowed to grow at 37°C for 16-18 hours to achieve 40-50% confluence before the medium was replaced with 1.5 ml of pre-warmed MEM+10% FCS. After 60 minutes, 0.5 ml HBS (pH 7.4) containing the test substance was added dropwise to each well and the mixture swirled gently to give an even distribution. The cells were then incubated at 37°C in 5% CO_2 for four hours. At the end of this period the transfection medium was aspirated and cells were washed twice with ice-cold MEM+10% FCS. Cells were then incubated under the standard cell culture conditions for 48 hours in 2.0 ml of complete medium before β-galactosidase activity was quantified.

4.3.1. Transfection of B16 cells using streptavidin-poly-L-lysine-DNA complexes.

Unless otherwise stated B16 melanoma cells were transfected using streptavidin-poly-L-lysine complexes prepared at a polypeptide-to-DNA mass ratio of 0.63:1 using the method described in section 3.8. It has previously been established that at this mass ratio the movement of plasmid DNA was completely retarded (section 3.12.). In all cases the volume of buffer in which the delivery system was administered was adjusted to 0.5 ml.

4.4. Influence of chloroquine on gene transfer.

Transfections were performed in the presence or absence of 100 μM chloroquine using streptavidin-poly-L-lysine-DNA complexes containing 6 μg pRSVlacZ. To test the influence of the cationic carrier polymer, cells were also incubated with an equivalent mass of free pRSVlacZ. Chloroquine (Sigma) was prepared as a 100 mM solution in water, sterilised by passing through a 0.2 μm filter, and stored at -20°C. Immediately before use, this solution was diluted to 10 mM with HBS (pH 7.4) and a

20 µl aliquot added into the medium. The plate was then swirled and complexes or plasmid DNA added to the cells. Cells were then treated as in section 4.3.1.

4.4.1. Dose response effect of chloroquine on gene transfer.

The experimental procedure in this case was as described in section 4.4. except that the concentration of chloroquine was varied. Immediately prior to introducing streptavidin-poly-L-lysine complexes, aliquots of the diluted chloroquine solution were added into the medium to give concentrations of 25, 50, 75, 100 and 150 µM. Cells were then treated as described in section 4.3.1.

4.5. Influence of polypeptide concentration on transfection efficiency.

The transfection procedure was as described in section 4.3. Complexes containing a fixed mass of pRSVlacZ (6 µg) and 0, 0.8, 1.4, 2.2, 2.6, 3.2, 3.8, 4.2, and 5.6 µg streptavidin-poly-L-lysine (calculated as free polylysine) were formed in a final volume of 0.5 ml HBS. Transfections were carried out in the presence of 100 µM chloroquine.

4.6. Effect of serum and time of harvesting on transfection efficiency.

In these experiments, cells were transfected using a method modified from that described in section 4.3.1. Cells were grown overnight in complete medium as previously described. One hour before transfection the complete medium was removed and the cell monolayer washed twice with either MEM+10% FCS or serum free MEM. The cells were then incubated with 1.5 ml of the appropriate medium for 60 minutes before the test substance was added. Transfections were performed in the absence or presence of serum (final concentration = 7.5% $^{v}/_{v}$) for the duration of the incubation period. Transfections were carried out in the presence of 100 µM chloroquine. Following a 4 hour exposure to DNA cells were treated as described in section 4.3.1. Cells were harvested 24, 48 and 72 hours after transfection.

4.7. Optimal DNA dose for transfection.

Cells were transfected using a protocol modified from that described in section 4.3.1. One hour before addition of complexes, growth medium was aspirated from each well and replaced with 1.38 ml of pre-warmed MEM+10% FCS. Cells were then incubated with 0, 125, 250, 375, 500, and 625 µl aliquots of the complex suspension which corresponded to doses of 0, 1.5, 3.0, 4.5, 6.0, and 7.5 µg pRSVlacZ respectively. The volume of medium was then adjusted to 2.0 ml with HBS (pH 7.4). Cells were then treated as described in section 4.3.1.

4.8. DNA stability in serum.

Streptavidin-poly-L-lysine-DNA complexes were prepared as described in section 3.8. Six micrograms of free DNA in 500 µl HBS, or complexes containing an equivalent mass of pRSVlacZ, were incubated for 4 hours at 37°C with 1.5 ml of MEM or MEM+10% FCS. The influence of nuclease activity on DNA stability was assessed by substituting the complete growth medium with MEM+10% FCS which had been heat treated at 56°C or 90°C for 30 minutes. After incubation of the DNA, a 40 µl aliquot was removed from each sample and mixed with 10 µl gel loading buffer. Thirty two microlitres of this mixture were analysed on a 1% agarose gel.

4.8.1. Availability of complexed DNA to enzymatic degradation.

Bovine pancreatic DNase I (Sigma) was dissolved at a concentration of 0.25 units/µl in 0.15 M NaCl/50% glycerol and stored at -20°C according to the method described by Sambrook *et al,* (1989). Samples containing 6 µg of pRSVlacZ or streptavidin-polylysine-DNA complexes were prepared in 500 µl HBS. To each sample 5 µl 1M $MgSO_4$ was added to give a final concentration of 10 mM Mg^{++}. Each mixture was then divided into two samples and 4 units DNase I was added to one of the two samples. The mixtures were incubated for 10 minutes at 4°C. Then a

40 µl sample was removed and mixed with 10 µl gel loading buffer before 10 µl aliquots were analysed on a 1% agarose gel.

4.9. Association of polypeptide-DNA complexes with B16 melanoma cells.

Analysis of the kinetics of association of a positively charged polypeptide-DNA complex with B16 melanoma cells was analysed using flow cytometry. Fluorescein labelled poly-L-lysine(219)-pRSVlacZ complexes were prepared at a DNA-to-polypeptide mass ratio of 0.6:1 as described in section 2.7.2. Cells were then incubated with these positively charged complexes for 30, 60, 120, 180 and 240 minutes at 37°C in the presence of 100 µM chloroquine. At the appropriate time point, adherent cells were washed twice with 1.0 ml of PBS and then removed using 0.02% $^{w}/_{v}$ EDTA. Cells were transferred to a 1.5 ml microcentrifuge tube, collected by centrifugation, washed and resuspended in 1.0 ml of PBS. Cell fluorescence intensity, relative to control cells, was measured at each time point as described in section 4.10. Comparison of the association of complexes with cells over time was made by comparing the mean fluorescence intensity after correction for changes in background fluorescence.

4.10. Flow cytometry.

Flow cytometry was used in the analysis of uptake of fluorescein labelled polymer-DNA complexes (section 4.9.) and in quantitative analysis of transfection efficiency (section 4.2.5.). In each case, quantitative fluorescence analysis of cell populations was performed using Becton Dickinson FacsVantage at an emission wavelength of 530 (± 15) nm following excitation at 488 nm using an argon ion laser. A minimum of 10^4 cellular events were analysed for each sample.

4.11. Use of Histone H1 as a cationic carrier polypeptide.

Histone H1-pRSVlacZ complexes were formed at polypeptide-to-DNA mass ratios between 0.63 and 2.3:1 and incubated with B16 melanoma cells according to

the transfection protocol described in section 4.3. Transfection efficiency of histone H1 was also compared with the optimised streptavidin-poly-L-lysine formulation on the same cell population. After 48 hours β-galactosidase activity was monitored in cell extracts using ONPG and expression measured at the cellular level using flow cytometry.

RESULTS

4.12. Requirement for chloroquine during transfection.

Cotten *et al,* (1990) have been successful in transfecting cells from the K-562 erythroleukemia line using plasmid DNA complexed with transferrin-polylysine. However, in order to achieve expression of the β-galactosidase reporter gene in more than a few cells of the population, the presence of chloroquine was required during the transfection period. Similar results were also obtained in the transfection of B16 melanoma cells with streptavidin-poly-L-lysine-DNA complexes (polypeptide:DNA (μg/μg)=0.63:1). When the complex alone was used to transfect cells, analysis indicated less than 2 milliunits of β-galactosidase activity per mg of soluble protein in each extract (Fig. 4.1.). Additionally, X-gal staining showed few or no positively stained blue cells (Fig. 4.2.b). Following the inclusion of chloroquine in the transfection medium, to a final concentration of 100 μM, a significant increase in β-galactosidase activity was demonstrated with analysis of cell extracts showing 416.6 ± 72.5 milliunits of enzyme activity per mg of soluble protein. A corresponding increase in the number of cells stained blue was observed with approximately 10% of cells expressing the enzyme (Fig. 4.2.c). In the absence of chloroquine gene transfer was 200 fold less efficient. However, transfection of cells with naked plasmid DNA in the presence of chloroquine did not produce significant levels of expression. Levels of endogenous β-galactosidase activity in this cell line were low and not increased by chloroquine treatment.

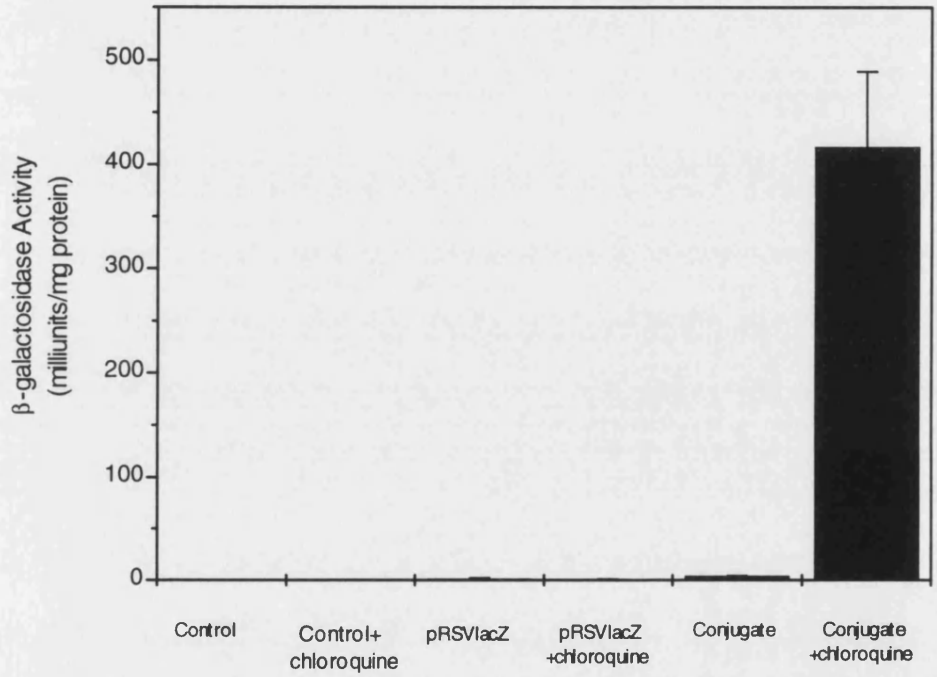


Figure. 4.1. Influence of chloroquine on transfection efficiency. B16 melanoma cells were transfected with plasmid DNA or streptavidin-poly-L-lysine-pRSVlacZ complexes in the presence or absence of 100 μM chloroquine. β-galactosidase activity was determined after 48 hours. Data is the mean ± SEM for three replicate transfections.

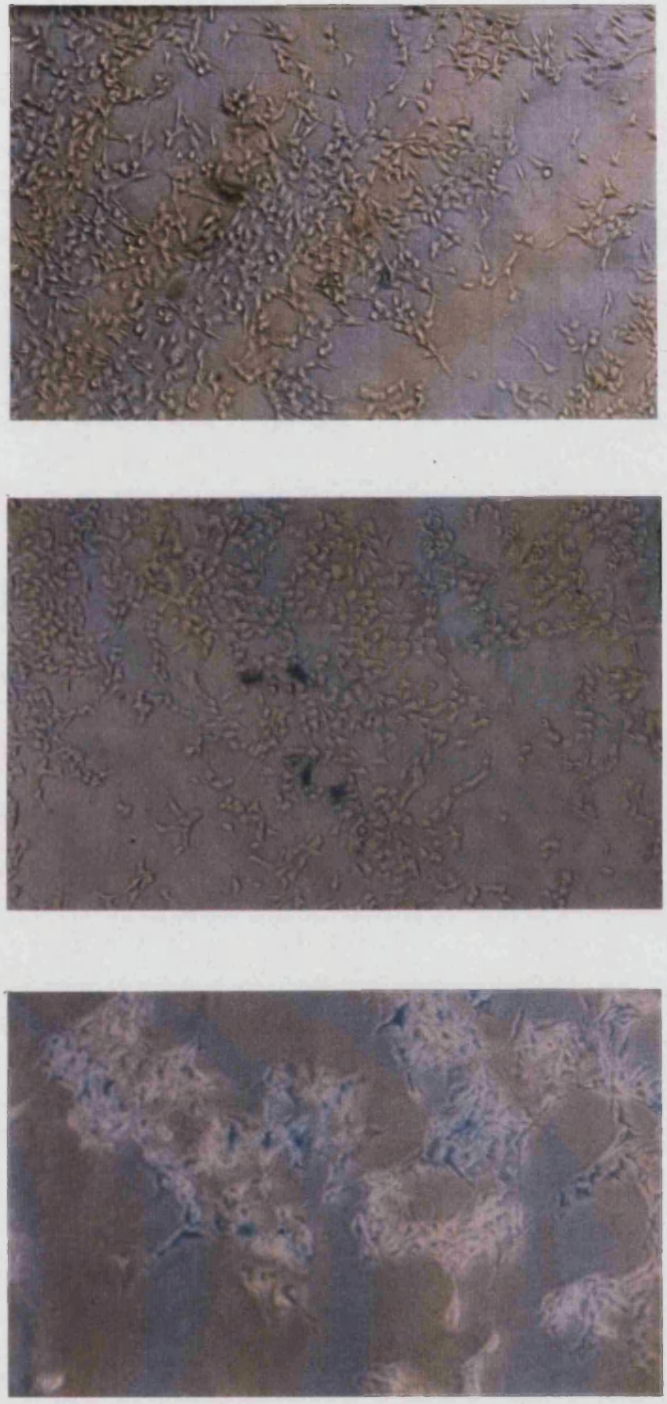


Figure. 4.2. *In situ* analysis of β-galactosidase activity in B16 murine melanoma cells after incubation with complexes. (x 200) (a) Control. (b) Complex only. (c) Complex with 100 μM chloroquine. Cells expressing β-galactosidase are stained blue.

4.13. Dose response effect of chloroquine on transfection efficiency.

The concentration of chloroquine used in experiment 4.4. (100 μM) was that detailed in the literature for transfection of HepG2 human hepatoma cells (Monsigny *et al*, 1994). The data shown in Figure 4.3.a illustrates the relationship between chloroquine dose, β-galactosidase activity and protein synthesis in B16 melanoma cells following simultaneous addition of 6 μg of complexed pRSVlacZ-DNA. An increase in relative β-galactosidase activity was seen at chloroquine concentrations of 25 μM and greater. As the concentration of chloroquine in the transfection medium increased an inhibition of protein synthesis was also produced leading to a corresponding reduction in total cell number per well and in the protein content of cell extracts. Analysis of total enzyme activity (Fig. 4.3.b) confirmed the optimal dose as 100 μM and showed the increase in relative enzyme activity was not simply a result of the reduction in the level of cell viability. At concentrations above 100 μM both relative and total β-galactosidase activity decreased, although little change in the total protein content of each well was observed.

4.14. Influence of complex polypeptide composition on transfection efficiency.

Various ratios of streptavidin-poly-L-lysine and pRSVlacZ were tested to determine the optimum formulation for gene transfer. Moreover, using the gel mobility shift assay shown in Figure 3.7. transfection efficiency could be related to the extent of DNA complexation. Maximum gene expression was achieved when 3.8 μg of streptavidin-poly-L-lysine was complexed with 6 μg pRSVlacZ (Fig. 4.4); at this ratio DNA was completely complexed (Polypeptide:DNA;(+/-) = 1.6). Transfection of cells with the 0.23:1 formulation, where DNA was significantly but not completely complexed, failed to increase expression above endogenous levels. Theoretical calculations showed that these complexes exhibited a net negative charge (Polypeptide:DNA;(+/-) = 0.6). Levels of β-galactosidase activity above those of the control were only seen when the polypeptide-to-DNA ratio was increased to 0.43:1

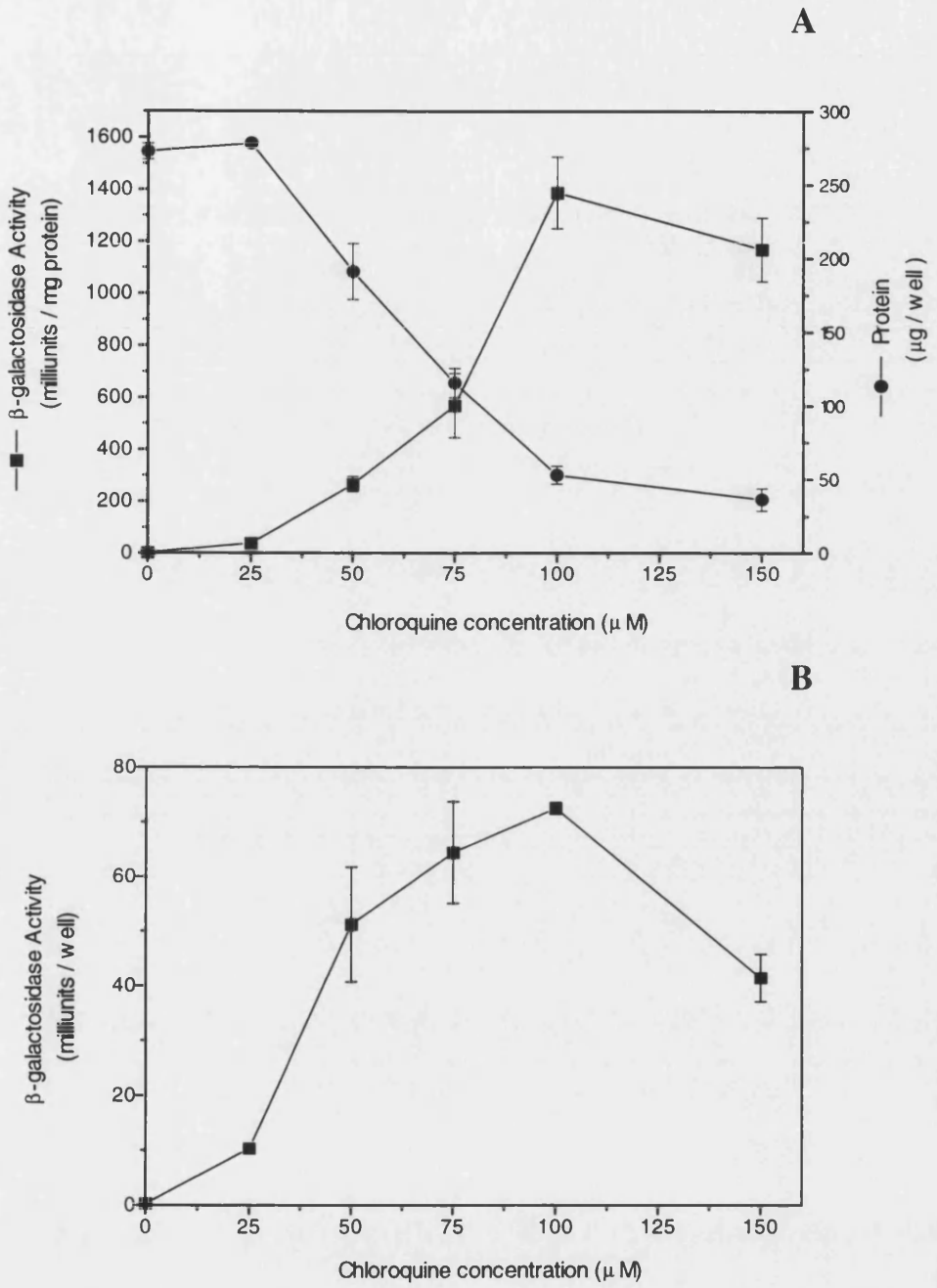


Figure. 4.3. Influence of chloroquine on the efficiency of transfection. Cells were transfected in the absence and presence of 25, 50, 75, 100 and 150 µM chloroquine. The effect of chloroquine on relative β-galactosidase activity and cell protein levels is described in Panel A. Total β-galactosidase activity/well is shown in Panel B. The results are the mean values from two replicate transfection plates. Bars indicate range.

which corresponded to a charge ratio of 1.1 (+/-). At mass ratios greater than 0.63:1, the optimum, increasing the proportion of polypeptide within the formulation resulted in a decrease in β-galactosidase activity. However, this decrease in relative activity was not accompanied by a reduction in protein synthesis.

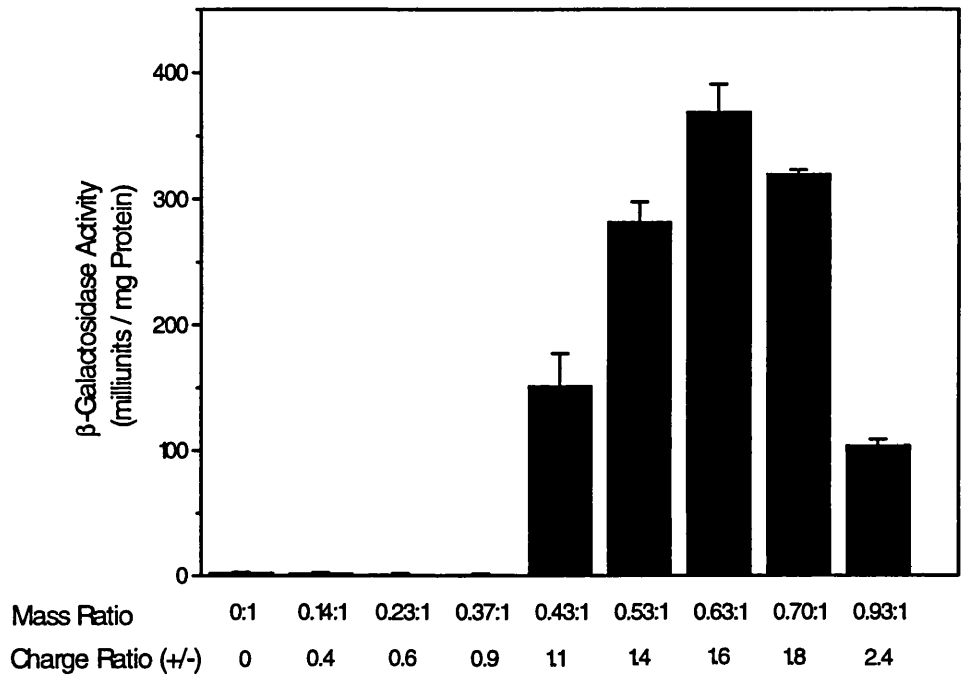


Figure 4.4. Influence of Polypeptide:DNA ratio on gene transfer. Complexes formed between pRSVlacZ and increasing quantities of streptavidin-poly-L-lysine were incubated with B16 melanoma cells at 37°C for four hours in the presence of 100 μM chloroquine. β-galactosidase activity was determined after 48 hours. Charge ratio of complexes was calculated as described in section 2.3. Each point represents the mean ± SEM for three replicate plates.

4.15. Effect of time of harvest and influence of serum on transfection efficiency.

Relative β-galactosidase activity was found to peak after 24 hours in the presence or absence of serum (Fig. 4.5.a). However, in the absence of serum a two fold higher

level of enzyme expression was seen at this time point. Over the following 48 hours a decrease in expression was evident with the equalisation of the activity observed in the samples transfected in the presence or absence of serum. The pattern of expression realised from these data conflicted with the general observation that transient expression of gene products reaches a maximum after 48 to 72 hours. However, if the data are not adjusted for the quantity of total protein in each sample, an indirect measure of cell number, then enzyme activity is lowest after 24 hours but increases over the remainder of the experimental period. After 72 hours the experiment had to be stopped as the cells had reached confluence.

4.16. Stability of polypeptide-DNA complexes in the transfection medium.

Assessment of carrier mediated protection was determined following incubation of plasmid DNA or streptavidin-poly-L-lysine-DNA complexes in transfection medium. Analysis of stability was performed using agarose gel electrophoresis and a representative gel is presented in Figure 4.6. The incubation of plasmid-pRSVlacZ in cell growth medium containing either native serum or serum heat denatured at 56° C for 30 minutes, resulted in rapid degradation. In both cases (Lane 4 and 5) degradation was essentially complete at the end of the four hour incubation period with only a small quantity of linearised DNA present in Lane 4. However, plasmid DNA incubated in the absence of serum (Lane 3) or serum heat denatured at 90°C for 30 minutes (Lane 6) showed migration patterns similar to control DNA (Lane 2). Complexed DNA, incubated for four hours in MEM (Lane 7) or in MEM+10% FCS (Lane 8), was retained at the top of the gel indicating that the DNA remained bound to the conjugate under the simulated transfection conditions. A detailed examination of DNA mobility, following incubation of the complex in the presence of serum, failed to detect the low molecular weight species characteristic of degradation (Lane 8).

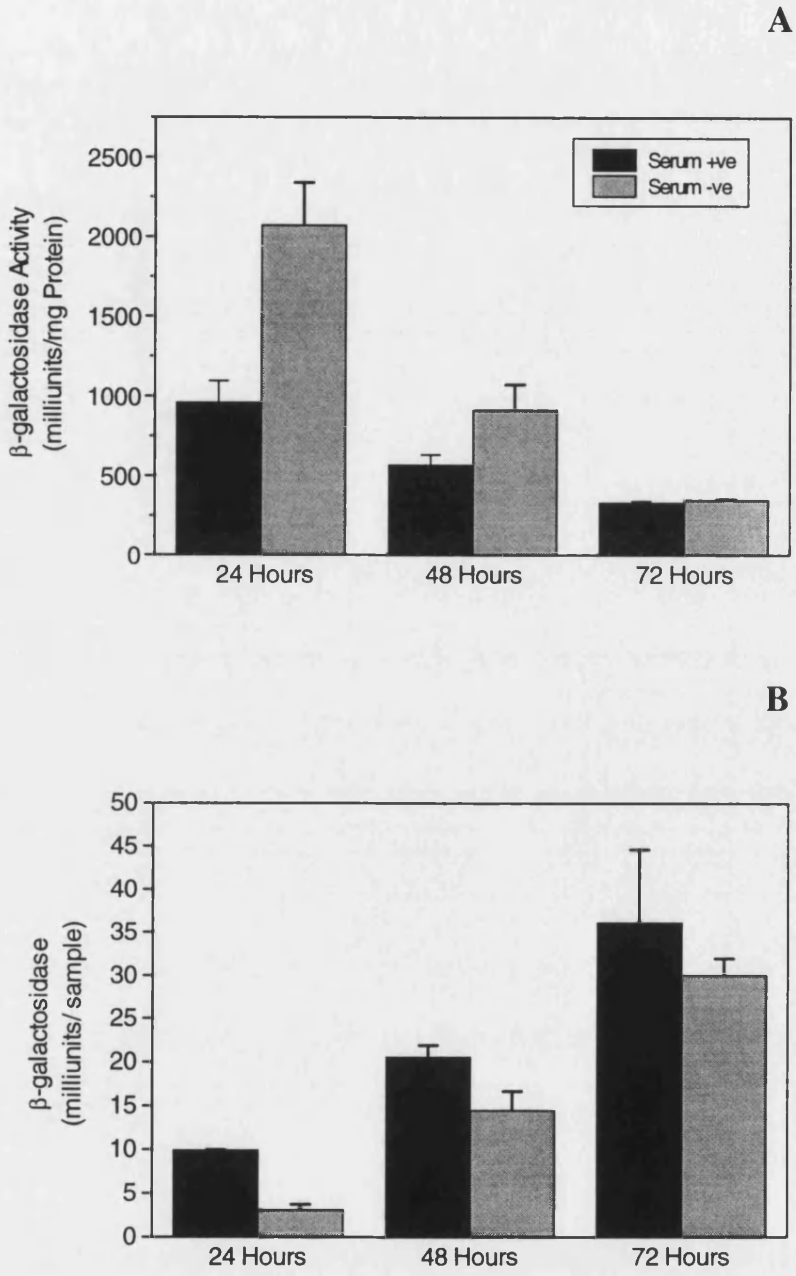


Figure. 4.5. Effect of serum and time of harvest on transfection efficiency. Transfections were performed with 6 µg pRSVlacZ in the presence or absence of serum. Cells were harvested 24, 48 and 72 hours later and assayed for β-galactosidase activity. The data are presented as relative (Panel A) and total β-galactosidase expression (Panel B). Each point represents the mean ± SEM for three replicate plates.

4.17. Availability of complexed DNA to enzymatic digestion.

The susceptibility of complexed plasmid DNA to enzymatic digestion was assessed by exposure to bovine pancreatic deoxyribonuclease I. The presence of Mg^{++}, a co-factor for the enzyme, was required for activity. Figure 4.7. shows that over a 10 minute incubation period plasmid DNA was rapidly degraded by DNase at a concentration of 8 units/ml. Preliminary experiments showed that if the incubation time was increased to 120 minutes then DNA could not be detected within the gel; the extent of degradation being sufficiently great to prevent detection with ethidium bromide. As expected DNA complexed with streptavidin-poly-L-lysine at a polypeptide to DNA ratio of 0.63:1 was retained at the gel origin (Lane 5 and 6). After incubation with DNase (Lane 6) the complexed DNA retained a pattern of electrophoretic migration identical to that of the control (Lane 5). These findings confirm that pRSVlacZ is rapidly degraded in the presence of nucleases and that DNA within the polymer complex is unavailable for digestion.

4.18. Optimisation of the dosing schedule.

In initial experiments, cells were incubated with free or complexed DNA for four hours before the test substance was removed and the transfection medium replaced. The effect of changing the length of incubation on β-galactosidase activity is illustrated in Figure 4.8. After incubation of cells with the optimised complex for 60 minutes, a small increase in enzyme activity above background levels was detected. After this time point relative β-galactosidase activity continued to increase throughout the six hour experimental period. Total β-galactosidase activity per well is optimal after 4 hours with a small decrease in enzyme expression produced on extending the incubation period to six hours. The rise in relative activity after 4 hours is, therefore, a reflection of the increased cytotoxicity produced by prolonged exposure to chloroquine rather than higher gene expression. Toxicity was evident from a significant decrease in the protein content of samples ($p<0.05$, *t* test unpaired).

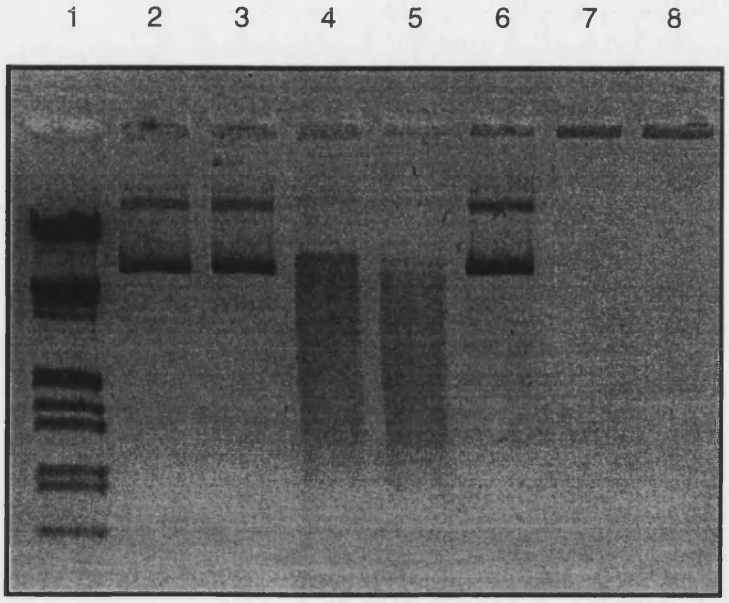


Figure. 4.6. Stability of pRSVlacZ and DNA-polypeptide complexes incubated in transfection medium. Free DNA and complexes were incubated at 37°C for four hours under the conditions described below, analysed on a 1% agarose gel and visualised using ethidium bromide.

Lane 1, λ DNA *EcoRI/Hind* III; **Lane 2**, pRSVlacZ; **Lane 3**, pRSVlacZ (MEM); **Lane 4**, pRSVlacZ (MEM+10% FCS); **Lane 5**, pRSVlacZ (MEM+10% FCS-heat treated at 56°C); **Lane 6**, pRSVlacZ (MEM+10% FCS-heat treated at 90°C); **Lane 7**, Streptavidin-poly-L-lysine-pRSVlacZ complex (MEM); **Lane 8**, Streptavidin-poly-L-lysine-pRSVlacZ complex (MEM+10% FCS).

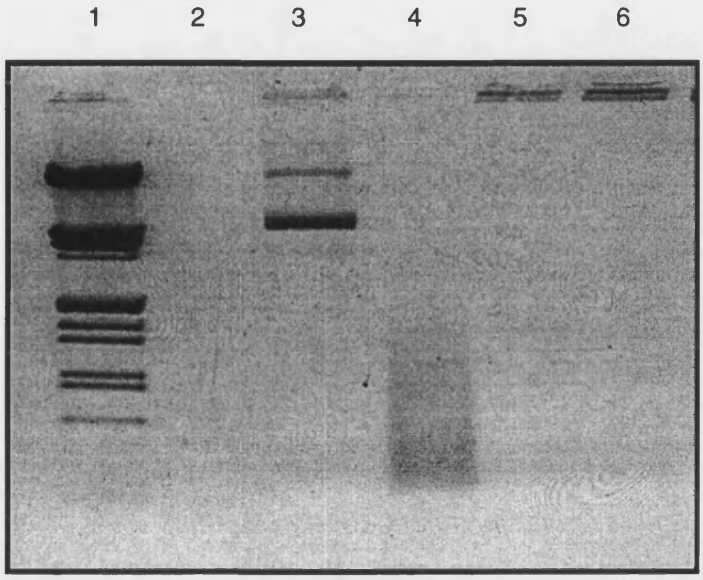


Figure 4.7. Availability of complexed DNA to enzymatic digestion. pRSVlacZ DNA or pRSVlacZ DNA complexed with streptavidin-poly-L-lysine was incubated with bovine pancreatic DNase I as described in section 4.8.1. and analysed on a 1% agarose gel.

Lane 1, *EcoRI/Hind*III λ DNA; **Lane 2**, Empty; **Lane 3**, pRSVlacZ; **Lane 4**, pRSVlacZ+DNase; **Lane 5**, Streptavidin-poly-L-lysine-pRSVlacZ complex; **Lane 6**, Streptavidin-poly-L-lysine-pRSVlacZ complex+DNase.

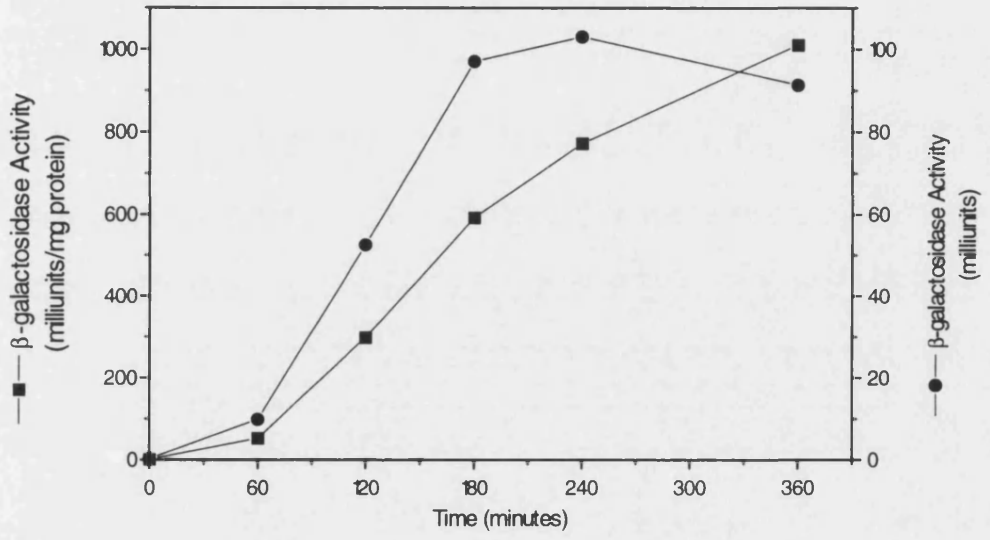


Figure. 4.8. Gene transfer as a function of time. Cells were transfected with 6 µg pRSVlacZ DNA complexed with the optimal ratio of streptavidin-poly-L-lysine. After incubation in the presence of 100 µM chloroquine for 60, 120, 180, 240 and 360 minutes, cells were washed twice with ice-cold fresh medium and incubated for a total of 48 hours. β-galactosidase activity was then determined. Results shown are the mean value from two replicate transfection plates.

4.19. Optimisation of DNA dose.

Transfection efficiency in B16 melanoma cells is dependent on the dose of DNA administered to the cells. Figure 4.9. shows that as the amount of DNA added to the cells is increased from 1.5 μg to 6 μg a 2.5-fold increase in relative β-galactosidase activity was produced. However, higher dose levels produced an apparent inhibitory effect with a 2-fold reduction in β-galactosidase activity when the dose was increased to 7.5 μg. The highest level of enzyme expression was observed from a 6 μg dose of pRSVlacZ.

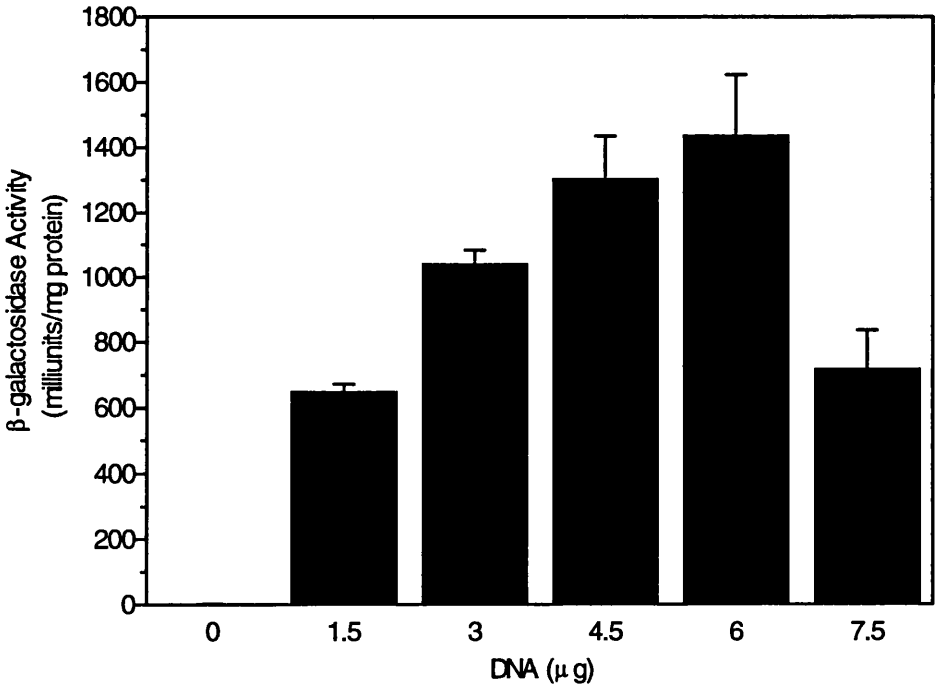


Figure 4.9. Dependence of β-galactosidase activity on DNA dose. Using the optimised streptavidin-poly-L-lysine-DNA formulation (described in section 4.14.). B16 melanoma cells were incubated with 0-7.5 μg of pRSVlacZ for 4 hours in the presence of 100 μM chloroquine. After 48 hours cell extracts were analysed for β-galactosidase activity. Data represent the mean ± SEM for three replicate plates.

4.20. Cell association of polycation-DNA complexes.

To assess the kinetics of cationic complex uptake, flow cytometry was used to follow the association of a fluorescein labelled poly-L-lysine-pRSVlacZ complex with B16 melanoma cells. The interaction of these complexes with cells over the four hour experimental period is evidenced by the shift in the fluorescence histograms to the right (Fig. 4.10.). An increase in relative fluorescence intensity (mean fluorescence intensity corrected for background), was also observed (Fig. 4.11.). Between one and three hours this increase was linear. A small rise in background fluorescence was also seen to over this period. Importantly, the change in relative fluorescence over the incubation period follows a profile similar to that described for the increase in total β-galactosidase expression over time. This analysis is indicative of a dependence of gene expression on the kinetics of gene delivery over the first three hours of the incubation period.

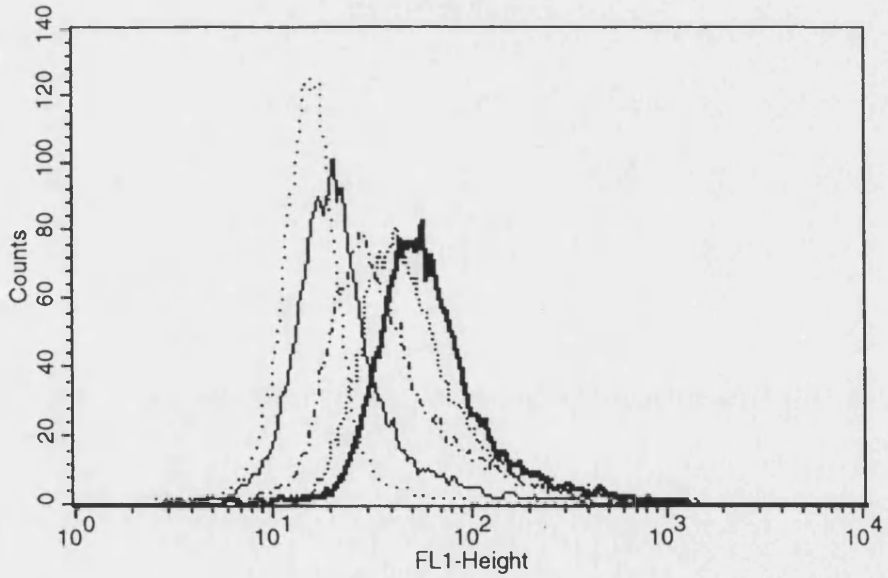


Figure 4.10. Complex association monitored by flow cytometry. Cells treated with a fluorescein labelled poly-L-lysine-DNA complex were analysed after 30 (····), 60 (---), 120 (-·-·-), 180 (- - -), and 240 (—) minutes. FL1=fluorescence intensity of channel 1, green fluorescence

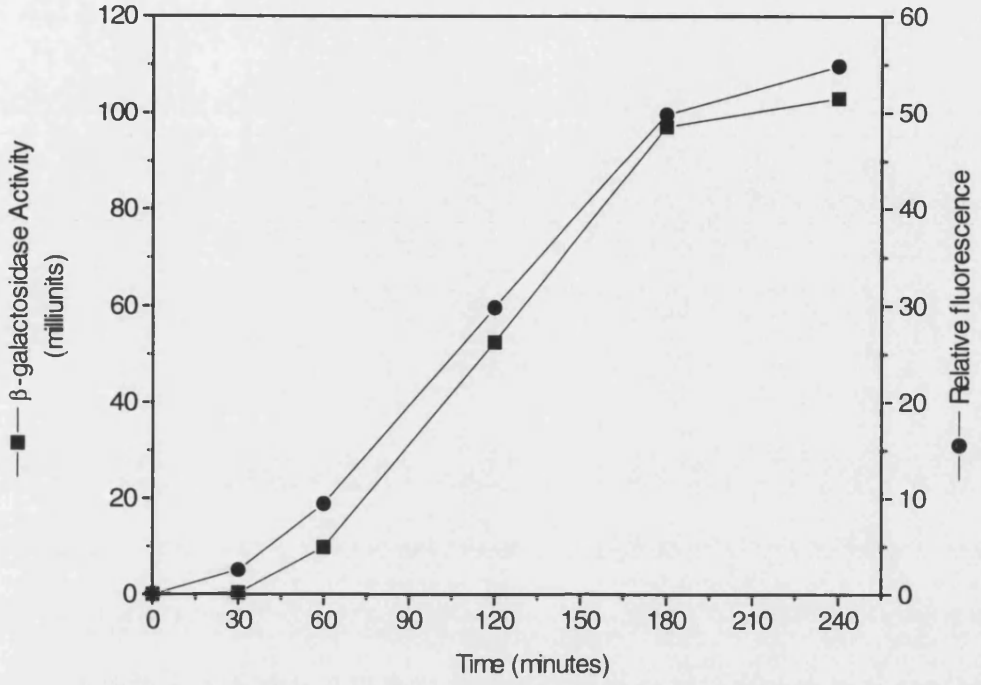


Figure 4.11. Uptake of complexes and gene expression as a function of time. Relative fluorescence intensity (●) and total β-galactosidase activity (■) were monitored following incubation of cells with FITC-poly-L-lysine-DNA complexes.

4.21. Transfection of B16 melanoma cells using Histone H1-DNA complexes.

Experiments to determine the optimum histone H1-to-DNA mass ratio for gene transfer to B16 cells were performed in the presence of 100 μM chloroquine (Fig. 4.12.). Under these conditions, the highest levels of β-galactosidase expression were seen following transfection with the 1.5:1 formulation. Similar to streptavidin-polylysine mediated delivery, gene expression was not seen until a critical polypeptide:DNA ratio was achieved. In addition, a significant decrease in transfection efficiency was observed with systems containing an excess of polypeptide; a three fold reduction in relative β-galactosidase expression was produced on increasing the mass ratio from 1.5:1 to 2.3:1. The transfection

efficiency of optimised histone H1 and streptavidin-polylysine complexes were also compared in this experiment. Histone-H1 complexes prepared at a polypeptide-to-DNA ratios of 1.2:1 and 1.5:1 produced levels of relative β-galactosidase activity in cell extracts which were significantly greater than with the optimal streptavidin-polylysine formulation ($p < 0.05$, *t* test unpaired). Indeed, the 1.5:1 histone H1 complex formulation, produced two-times the activity seen with the optimised streptavidin-poly-L-lysine formulation. A similar difference was also apparent on analysis of total activity.

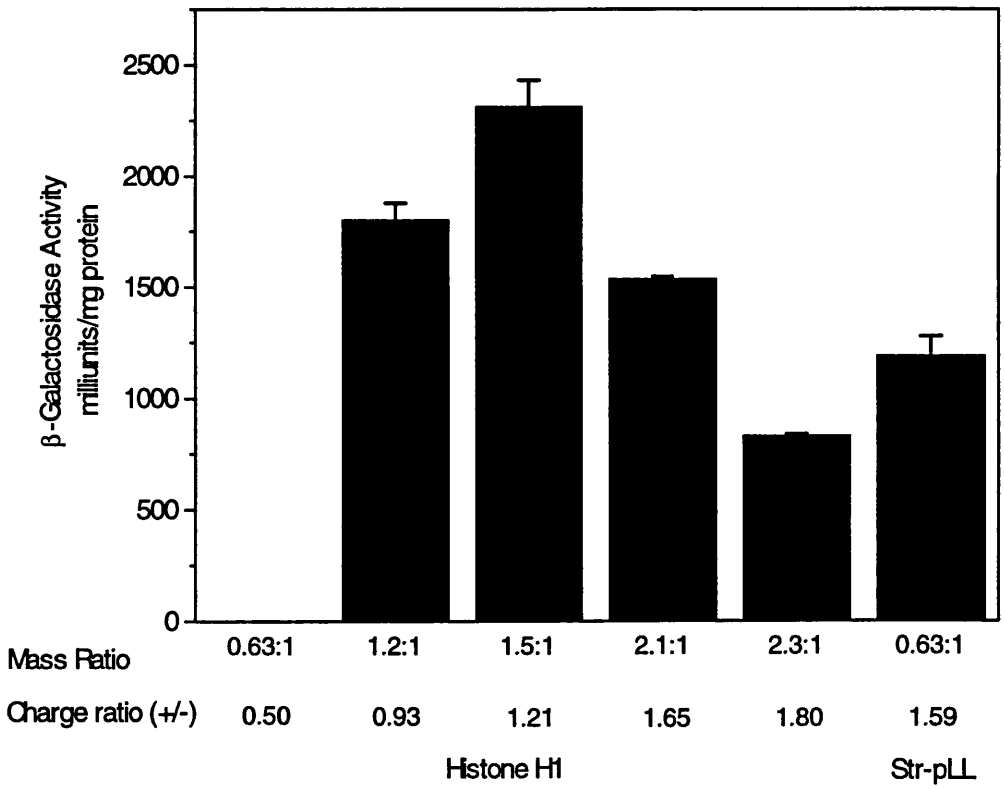


Figure 4.12. Influence of histone H1-to-DNA ratio in terms of mass and charge on gene transfer. The optimal streptavidin-poly-L-lysine:DNA formulation was also tested. Complexes were incubated with B16 melanoma cells for 4 hours in the presence of 100 μM chloroquine. Data represents the mean ± SEM for three replicate plates.

4.22. Analysis of β-galactosidase expression by flow cytometry.

Flow cytometry was used to discriminate between $LacZ^+$ and $LacZ^-$ cells following transfection of cells using pRSVlacZ-DNA. Control cells incubated with FDG (Fig. 4.13.b) showed a small increase in relative fluorescence above that described for an unstained cell population (Fig. 4.13.a). This staining was associated with a low level of endogenous β-galactosidase activity. Cells transfected with pRSVlacZ developed a fluorescence distribution pattern distinct from that of the controls. In Figure 4.13.c , a major peak with fluorescence intensity higher than the autofluorescent control and a second peak with stronger fluorescence were observed. As the major histogram peak exhibited a fluorescence pattern similar to that seen for the controls, and both $LacZ^+$ and $LacZ^-$ cells were detected on staining pRSVlacZ transfected cultures with X-gal, then it was thought likely that cells in this peak represented a negative population. The shift in the negative peak probably resulted from free fluorescein leaking from highly expressing cells into $lacZ^-$ cells during the initial hypotonic shock (M. Roederer, personal communication). Therefore, in determining the proportion cells expressing β-galactosidase, fixed markers applied to the autofluorescent control could not be used on transfected cell populations. Rather, a marker (M1) was placed manually to include all cells in the second (+ve) peak and exclude negative cells (-ve).

4.23. Comparative analysis of transfection efficiencies by flow cytometry.

The results of studies to quantitate the ability of optimised streptavidin-polylysine and histone H1 complexes to deliver plasmid DNA are presented in Table 4.1; the relative proportion of cells which were $lacZ^+$ were compared to those that were $lacZ^-$. Transfection of cells with a streptavidin-polylysine system generated β-galactosidase expression in 16.4 ± 0.4 % of the total cell population. However, by delivering the 6.0 μg dose of pRSVlacZ in Histone H1 complexes it was possible to increase the proportion of the cell population expressing β-galactosidase to 26.4 ± 0.92 %.

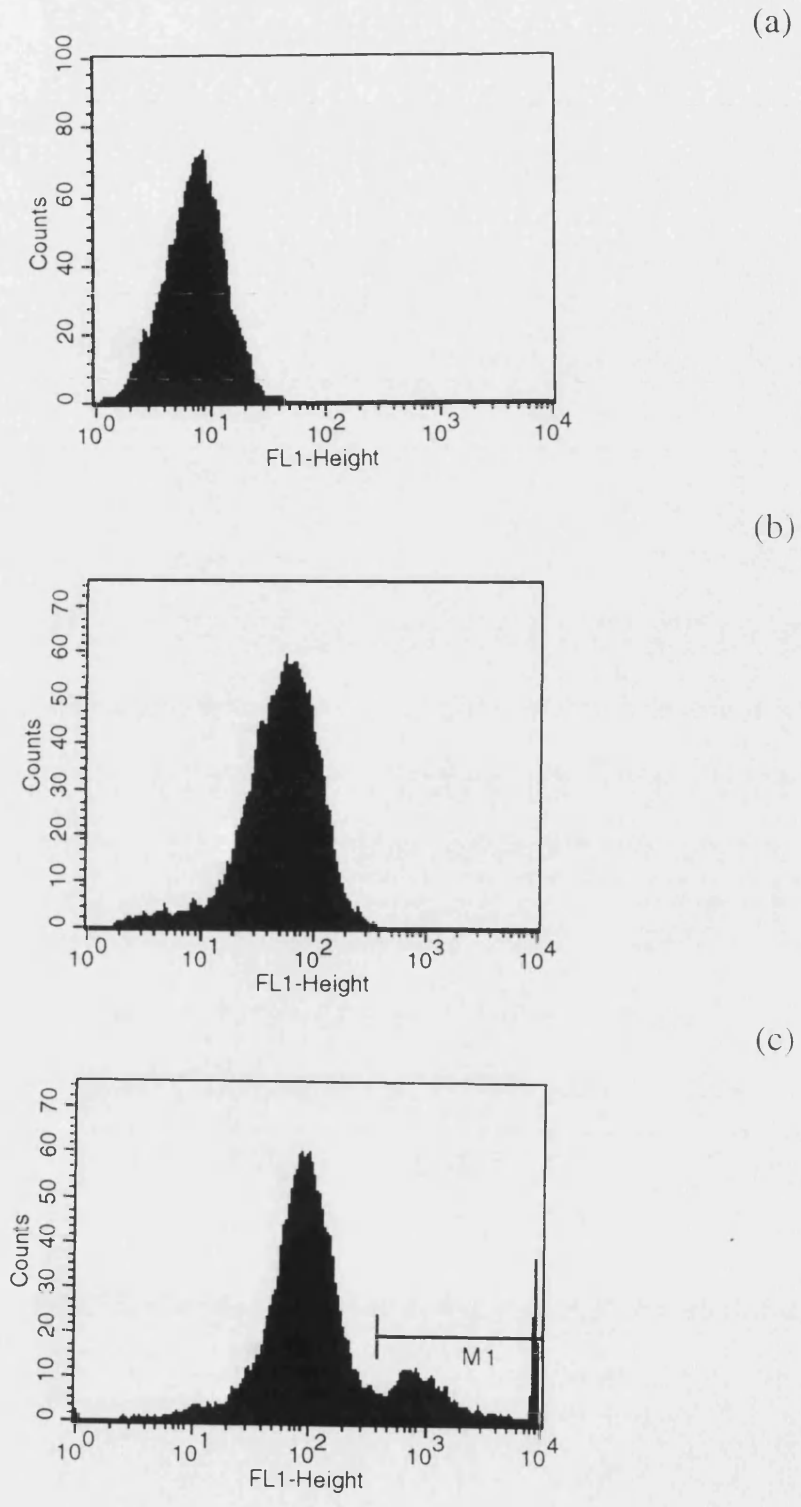


Figure 4.13. Detection of B16 melanoma cells expressing β-galactosidase using flow cytometry. Cells were transfected using streptavidin-poly-L-lysine in the presence of 100 μM chloroquine. (a) Control cells (b) Control cells; FDG stained (c) pRSVlacZ transfected cells.

Formulation	Events	Cells Positive(±SD)	% Positive(±SD)	n
Streptavidin-polylysine	10000	1612 ± 44	16.1 ± 0.4	3
Histone H1	10000	2637 ± 93	26.4 ± 0.92	3

Table 4.1. Expression of β-galactosidase in B16 cells following transfection using Streptavidin-polylysine and histone H1. The proportion of the cell population expressing the enzyme was analysed using the fluorescent substrate fluorescein-β-D-galactopyranoside.

4.24. Discussion.

4.24.1. Gene transfer using streptavidin-polylysine: The influence of chloroquine.

When streptavidin-poly-L-lysine complexes were incubated with B16 melanoma cells without chloroquine, appreciable expression did not occur. This was demonstrated by the lack of cells staining positive following treatment with X-gal and the low levels of total β-galactosidase activity found in cell extracts. To determine whether expression was limited by the degradation or trapping of DNA within the endosomal/lysosomal trafficking pathway, cells were incubated with 100 μM chloroquine during transfections. Under these conditions significant levels of β-galactosidase expression could be achieved with a 200-fold enhancement of expression over the activity produced by the DNA complex alone. These results are in general agreement with the work of Cotten *et al,* (1990) who described the effect in the K-562 erythroleukemia cell line and Midoux *et al,* (1993) who used chloroquine to enhance gene transfer in HepG2 human hepatoma cells. However, chloroquine treatment of alveolar macrophages transfected with polylysine-IgG-DNA complexes produced a more modest (1.6-fold) increase in gene expression (Rojanaskul *et al,* 1994). An absolute analysis of the data from the later study was not possible as the authors used a non-standard assay procedure and the levels of transfection within the total population were not described.

Improvements in gene transfer efficiency are, however, only produced with relatively high concentrations of chloroquine (Zenke *et al,* 1990). Gene transfer efficiency in B16 melanoma cells is seen to increase at chloroquine concentrations greater than 25 μM with maximum levels of β-galactosidase produced at a concentration of 100 μM. The requirement for such high concentrations of chloroquine is inconsistent with the traditional mechanism by which chloroquine is thought to enhance gene expression. The weakly basic molecule was thought to increase the survival of transferred DNA by increasing the pH of the lysosomes and

inhibiting the activity of nucleases. However, inhibition is produced at sub-toxic chloroquine concentrations (≈10μM; Dean *et al,* 1984) which are much lower than those required to increase gene expression. An alternative hypothesis, postulated by Zatloukal *et al,* (1992), is that on accumulation in the endosome chloroquine acts osmotically, vacuolarising and finally disrupting the endosome with release of complexes into the cytosol. This hypothesis was confirmed in K-562 cells, where the absence of Na^+,K^+-ATPase regulation of endosomal acidification allows accumulation of exceptionally high levels of the drug. In mammalian cells the Na^+-K^+ transporters are responsible for the maintenance of the pH gradient and as a consequence of their absence the pH of the endosomal compartment reaches unusually low values (Sipe *et al,* 1991). Consequently, virtually 100% of the cells express the reporter gene following transfection. This contrasts with the 7-10% of B16 melanoma cells expressing β-galactosidase following a similar treatment. The disadvantage of the chloroquine procedure was the appearance of dose related cell toxicity at concentrations greater than 25 μM; this being the concentration above which gene transfer was augmented. Cytotoxicity was particularly evident on the prolonged exposure of cells to high concentrations of the drug. Therefore, increasing the time of the incubation period did not serve to improve the efficiency of gene transfer.

The influence of chloroquine on gene expression provides an insight into the mechanism of cationic-polypeptide polynucleotide delivery. Indeed, the dependence of expression on the lysosomotropic agent chloroquine suggests that DNA complexed with polycations is delivered to the nucleus via the endosome-lysosome trafficking pathway. Moreover, the incorporation of complexes into endosomal compartments is consistent with complexes entering cells via a process of endocytosis rather than directly through the plasma membrane. A similar uptake mechanism has been proposed for cationic liposomes (Legendre and Szoka, 1992).

4.24.2. Complex composition influences the uptake of DNA.

Since underivatised polymers have been used successfully for the delivery of plasmid DNA, then it may be assumed that some mechanism exists for the binding of these complexes to the cell surface and internalisation of DNA. Examining β-galactosidase expression as a function of the polypeptide content of complexes gave an indication of this mechanism. Increasing the streptavidin-poly-L-lysine content of formulations only produced competent delivery systems at polymer-to-DNA mass ratios greater than 0.37:1. Taking into account that a streptavidin-poly-L-lysine(219) molecule possesses 218 remaining cationic charges, and that pRSVlacZ is a plasmid of 7.8 kb, electroneutrality in complexes formed between str-pLL and pRSVlacZ is obtained when a plasmid molecule is covered by 82 str-pLL molecules. Therefore, by increasing the polypeptide-to-DNA ratio from 0.37:1 (Polypeptide:DNA; (mol/mol) = 75:1) to 0.43:1 (Polypeptide:DNA;(mol/mol) = 90:1) a change in the charge density from net negative (Polypeptide:DNA;(+/-) = 0.9) to net positive (Polypeptide:DNA;(+/-) = 1.1) is produced. These data suggest plasmid uptake is dependant on the charge of a complex. By further increasing the polypeptide to DNA mass ratio to 0.63:1 an optimum level of β-galactosidase expression was achieved. Perhaps surprisingly, the decrease above this optimum appeared unrelated to toxicity. Therefore, it is proposed that the reduction in β-galactosidase expression reflects a change in delivery efficiency that is perhaps associated with changes in complex conformation.

4.24.3. Optimisation of DNA dose.

Using the optimised streptavidin-polylysine-DNA formulation described above, the effect of DNA dose on transient gene expression was evaluated. The highest levels of β-galactosidase activity were achieved following delivery of 6 μg DNA, although significant levels of expression were seen with a 1.5 μg dose of pRSVlacZ. DNA levels above the optimum appeared to be inhibitory although activity was not

completely abolished. If the studies by Felgner *et al*, (1987), using cationic liposomes, relate to this situation then it would appear that the quantity of DNA required to generate an optimal signal varies with the cell type. Therefore, it will probably be necessary to optimise the system for the transfection of individual cell lines. It is also noteworthy that optimal doses reported in the literature for *in vitro* transfection with systems based on cationic polypeptides have varied between 2 μg (Zenke *et al*, 1990) and 20 μg of DNA (Midoux *et al*, 1993)

4.24.4. Stability of cationic-polypeptide-DNA complexes.

The conditions to which DNAs are exposed in transfection experiments are generally hostile with substantial nuclease activity evident in serum and some intracellular compartments (e.g. lysosomes). Indeed, Felgner *et al*, (1987) showed that the transfection of mouse L cells with a DOTMA liposomal type delivery system was inhibited by serum containing medium. Serum stability is, therefore, likely to significantly influence the success of any attempt to use non-viral systems for gene transfer *in vitro* and *in vivo*. Studies with streptavidin-polylysine complexes have indicated that after 24 hours relative activity was significantly lower when transfections were performed in complete growth medium. This suggested potential instability of complexes with subsequent degradation of a fraction of the DNA dose. However, comparing Fig. 4.5.a and Fig. 4.5.b shows that corrections for the protein content of samples effectively raise the relative values of enzyme activity for the transfections performed without serum. If total activity is examined then the presence of serum appears to have a positive effect on the levels of expression. It is likely this paradox reflects the complexity of the model system in which the influence of serum on cell viability cannot be separated from any potential effect on the delivery system.

The stability of polypeptide-DNA complexes was, therefore, evaluated under conditions which simulated the extracellular environment encountered during *in vitro*

transfections. These studies confirmed earlier published data which showed oligodeoxynucleotides and double stranded DNAs to be readily degraded in culture medium containing serum (Wickstrom, 1986; Chiou *et al*, 1994). It is noteworthy that degradation is only arrested following heat treatment at 90°C and not following incubation at 56°C for the 30 minutes; the latter are the standard conditions used to prepare 'heat inactivated' serum. These results also appear to reflect the *in vivo* situation where DNA is degraded in a similar manner although the kinetics of the process appear to be more rapid (Kawabata *et al*, 1995). The level of deoxyribonuclease activity in human and calf serum has been shown to be similar (Cox and Gokcen, 1976). Following complexation with streptavidin-poly-L-lysine, DNA was protected for the duration of the transfection period. Additionally, the stability of complexed DNA in presence of deoxyribonuclease demonstrates that in this state nucleic acid is no longer available for enzymatic degradation. It is therefore apparent that DNA stability is, at least in part, caused by the conformational change induced by polylysine containing conjugates.

4.24.5. Time course of gene expression.

In vitro gene expression mediated by cationic polypeptide-DNA complexes was transient with the maximum level of relative activity observed after 24 hours. This closely parallels the expression pattern noted by Gao *et al*, (1993) following transfection of cotten rat airway epithelial cells with human transferrin-polylysine-DNA complexes. However, analysis of changes in total β-galactosidase activity with time gave a different pattern of expression to that described above for relative activity. Here, an increase in activity was observed over the 72 hour experimental period. These differences in expression patterns were thought to reflect the multiplication of non-transfected cells which effectively reduces the levels of relative activity over time. The transient expression of gene products in this system is not entirely unexpected as the DNA delivered in these experiments does not contain any

mechanism to enable long term gene transfer. However, using a cationic-polypeptide system Cotten *et al*, (1993) showed that the incorporation of a replicative episome into plasmid vectors could successfully be used to produce gene expression for several weeks *in vitro*. It is also possible to prolong gene expression following gene transfer to hepatocytes *in vivo* by surgical intervention (Wilson *et al*, 1992). The mechanism proposed for these events was the stabilisation of internalised DNA by partitioning into nuclear or cytoplasmic compartments. However, these results were in conflict with the general literature which suggests that if episomes are to persist in eukaryotic cells they must integrate into chromosomal DNA. These techniques may remove the necessity for repetitive dosing in correcting genetic defects. However, the permanent modification of cells may be ethically unacceptable.

4.24.6. Kinetic analysis of DNA delivery.

In assessing the capability of polymeric systems to deliver nucleic acids to mammalian cell lines, the kinetics of the process are of great interest. The association of complexes with B16 melanoma cells was followed with systems formulated with fluorescein labelled polylysine. Under the specific conditions described in section 4.9., the relative fluorescence of cell populations incubated with these complexes increased over the first three hours of the incubation period and then remained relatively constant. These data suggest that the cell association of charged polycation complexes occurs gradually and is relatively slow. Importantly, the relationship between β-galactosidase expression and incubation time showed a similar dependence on time. Therefore, in measuring these changes in relative fluorescence it is probable that the internalisation of the complexes, and associated DNA, was being monitored.

4.24.7. Histone H1 as a cationic polypeptide carrier.

Histones were examined as an alternative carrier system for both non-specific and receptor-mediated gene delivery as in low ionic strength solvents complexes with

DNA exist as soluble particles (Olins and Olins, 1971). Histone H1-DNA complexes exhibit similar morphology to those prepared with the synthetic homopolymer poly-L-lysine (Laemmli, 1975). Complexes constructed from a fixed mass of supercoiled plasmid DNA and increasing quantities of histone H1 showed a similar spectrum of transfection activity to streptavidin-polylysine in the presence of 100 μM chloroquine. However, if the optimum formulation of each of these delivery systems is compared then histone H1 produces significantly higher levels of β-galactosidase in cell extracts. Importantly, this increase reflects a greater proportion of the cell population expressing the enzyme rather than an increase in the level of gene product synthesised by each transfected cell.

A number of reasons may be postulated for the superiority of histone H1 in transforming B16 melanoma cells; changes in complex structure, nuclear localisation of histone based systems, and the efficiency of DNA release from the delivery vehicle. It is likely that changes in structure will significantly influence the uptake of complexes in cell systems. However as stated previously, electronmicrographs of complexes prepared with histone H1 and polylysine revealed structures of similar morphology. As the experimental conditions were similar to those for the preparation of streptavidin-polylysine then complexes of similar dimensions would be expected with this system. Perhaps of more importance is the targeted trafficking of the carrier polypeptide to the nucleus. The co-introduction of the non-histone high mobility group protein-one (HMG-1) and DNA in lipososmes has dramatically increased DNA accumulation in the nucleus (Kaneda *et al*, 1989). When BSA was used instead of HMG-1, DNA was located mainly in the cytoplasm. It is also possible that complexes containing histone H1 undergo facilitated transport into the nucleus. Indeed, the complete 21 kDa protein (Bonner, 1975), and proteolytic fragments of the C-terminus domain (Dingwall and Allan, 1984), accumulate in nuclei by an energy dependent process following interaction with cytoplasmic

binding factors (Breeuwer and Goldfarb, 1990). However, in discussing this mechanism we should consider that the channels in the nuclear pore complex, through which transport occurs are approximately 100 Å in diameter and may prevent the entry of large complexes (reviewed by Peters, 1986). A third factor, which is not discussed in the literature, is the relative efficiency of DNA release from the delivery system. From data presented by Rosenkranz *et al,* (1992) it appears that DNA remains bound to the carrier polycation in the nucleus. Therefore, in this study, DNA is perhaps more readily released from histone H1 than polylysine Indeed, Olins *et al,* (1969) have shown that significantly more energy is required to induce changes in polylysine-DNA complexes than in systems prepared with lysine rich histone fractions. In addition, mechanisms are known to exist which regulate the interactions between histone H1 molecules (Ogata *et al,* 1980). These systems play an important part in the formation of higher order structures in the polynucleosome strands (solenoids). Therefore, it is possible that these endogenous regulatory systems are able to release DNA from histone H1 more readily than from polylysine.

4.25. Summary.

In summary, factors which have been shown to determine the efficiency of gene transfer can be divided into those directly related to the formulation (cationic carrier, complex composition, stability, dosing schedule, DNA dose) and external influences (rate of internalisation and lysosomal degradation).

The polypeptide composition of formulations has a significant influence on gene transfer; uptake of DNA is only achieved at a specific polypeptide-to-DNA ratio and then reaches an optimum on addition of increasing quantities of streptavidin-polylysine. Using this data, formulations can be prepared for gene targeting which minimise uptake by non-specific effects. However, successful transfer and

expression of the β-galactosidase reporter gene also requires the presence of chloroquine. These facts support the hypothesis that the mechanism of uptake is related to charge. However, no firm conclusions can be drawn as to the influence of charge in this system since these data were inferred from theoretical calculations. These results provided the basis for the more detailed investigations presented in chapter 5. The function of the cationic carrier polymer in producing an effective delivery system also appears to extend to the protection of complexed DNA.

Chapter 5

Influence of cationic polypeptides on gene transfer.

It is apparent from data presented in this study and the published literature, that polycationic agents are effective in facilitating the entry of plasmid-DNA into cells in culture and *in vivo*. The majority of these studies have used polymers coupled to ligands which bind cell surface receptors. However, it is possible to envisage therapeutic situations, for example where the disease is compartmentalised (e.g. cystic fibrosis) or where unique receptors are not present on cells, when non-specific methods of gene transfer may be applicable. To this end, the formulation factors which may influence the efficiency of non-specific gene transfer have been investigated. Complexes were prepared from a range of structurally diverse polymers and their ability to transfer DNA assessed in relation to changes in structure, molecular weight and charge. In order to conclusively establish if non-specific gene transfer was related to charge, as suggested by experiments with streptavidin-polylysine (section 4.14.), electrophoretic light scattering was used to measure the zeta potentials of model polypeptide-DNA complexes. Physical characteristics of complexes were also examined by electron microscopy.

5.1. Influence of polypeptide-to-DNA ratio on the transfection efficiency of complexes prepared using poly-L-lysine.

Complexes containing 6 μg pRSVlacZ and increasing masses of poly-L-lysine (13), (127), (214) and (859) were formed in a final volume of 0.5 ml HBS using the method described in section 2.4. In general, systems with polypeptide-to-DNA mass ratios between 0.1:1 and 1.0:1 were used in transfections. However, for poly-L-lysine(13) mass ratios of up to 3.0:1 were tested. Transfections were carried out in the presence of 100 μM chloroquine.

5.2. Influence of polymer chain length on transfection efficiency.

The ability of polymers to deliver plasmid DNA was assessed using the test system described in section 4.2. Transfections were performed in triplicate in the presence of 100 μM chloroquine using poly-L-lysine(13), (127), (214) and (859). Formulations were prepared using the optimal polypeptide-to-DNA mass ratios determined in section 5.1. β-galactosidase activity was measured after 48 hours.

5.3. Alanine-lysine graft co-polymers as an alternative to polylysine carriers.

Complexes of DNA and the alanine-lysine graft co-polymers described in section 2.2.3. were prepared at polypeptide-to-DNA mass ratios between 1.2:1 and 2.9:1. Transfections were performed in the presence of 100 μM chloroquine as described in section 4.2. and β-galactosidase activity determined in cell extracts 48 hours later.

5.4. Stability of polylysine-DNA complexes of variable polymer compositions.

Poly-L-lysine(214)-DNA complexes of polypeptide-to-DNA mass ratios 0:1, 0.2:1, 0.4:1, 0.5:1, and 0.8:1 were prepared in 0.5 ml total volume of HBS. Each mixture was then divided into two samples and DNase I added according to the protocol described in section 4.8.1. Complexes were analysed on a 1% agarose gel.

5.5. Measurement of zeta potentials.

Zeta potentials of complexes formed between poly-L-lysine(214) and pRSVlacZ, at polypeptide-to-DNA mass ratios between 0.2:1 and 5.0:1, were determined using an electrophoretic light scattering technique (Brookhaven Instruments, Zetaplus). Formulations were prepared in both HBS and water. Application of a constant current across a cell containing the colloidal system results in the movement of charged particles toward the appropriate electrode. The velocity distribution of particles is then calculated from analysis of the Doppler shift of light scattered by the particles against an imposed reference beam (250 Hz). Scattered light was measured at an angle 15° from the incident. All glass and plasticware used in the preparation

of complexes and measurement of zeta potentials was washed three times with Milliq-grade water (prefiltered; 0.1 μm nylon membrane) to reduce particulate contamination. Water and HBS used in the dilution of polylysine and DNA solutions was also filtered before use. Complexes were formed as described in section 2.4. and incubated at room temperature for 30 minutes. A 1.6 ml volume of sample solution was then transferred to a freshly cleaned 3.0 ml disposable cuvette. Before five repeat measurements were made the cuvette was allowed to equilibrate in the instrument for five minutes to achieve a constant temperature of 25°C. The 250 Hz reference point was established between each reading.

5.6. Characterisation of polylysine-DNA complexes by electron microscopy.

The physical characteristics of polylysine(214)-DNA complexes formed at different polypeptide-to-DNA mass ratios were monitored by electron microscopy according to the method modified from Ennever *et al,* (1985). Complexes were prepared in water at polypeptide-to-DNA mass ratios of 0.4:1 (the optimum for transfection), 1.0:1 and 5.0:1. Immediately after preparation, DNA complexes (12 μg/ml) were applied to 200 mesh copper grids supported on a pioloform film. Samples were allowed to adsorb onto the grid over approximately two minutes, blotted and stained with 1% uranyl acetate (1 minute). The grids were then rinsed by floating on double distilled water (1 minute) to remove excess stain. Specimens were examined on a JEOL-1200EX II transmission electron microscope operating at 80kV.

Results.

5.7. Transfection activity of poly-L-lysine molecules with differing chain lengths.

In order to examine the relationship between transfection efficiency and polymer chain length, it was first necessary to determine the optimal complex formulation for each of the poly-L-lysine molecules to be tested. To this end, cells were incubated with complexes of variable polymer content according to the method described in

section 4.2. Levels of relative β-galactosidase activity were then measured after 48 hours and plotted as a function of the polymer-to-DNA mass ratio (Fig. 5.1.). For both poly-L-lysine(214) and (859) maximum gene expression was produced following transfection of cells with complexes prepared at a mass ratio of 0.4:1. However, with poly-L-lysine(127) the optimum occurred at the higher mass ratio of 0.5:1. This was true whether the transfection efficiency was expressed as relative β-galactosidase activity or total activity per well. Several formulations based on poly-L-lysine(13) and the alanine-lysine graft co-polymers were also tested, but these systems failed to increase β-galactosidase expression above endogenous levels (Fig. 5.2.). The optimised formulation for each competent polypeptide carrier was then used in a subsequent experiment to assess the influence of polymer chain length on gene transfer efficiency (Fig. 5.3.). Following delivery of 6 μg pRSVlacZ, gene expression facilitated by poly-L-lysine(127) complexes was two times greater than that produced with either poly-L-lysine(214) or (859). No significant difference ($p<0.05$, *t* test unpaired) in β-galactosidase activity was found in cell extracts following transfection of cells using poly-L-lysine(214) or (859). In this experiment, the rank order of polymer delivery efficiency was the same as that described in the optimisation study.

5.8. Stability of complexes of variable polylysine content.

A representative electrophoretic gel of poly-L-lysine(214)-DNA complexes incubated with DNase is shown in Figure 5.4. As expected, DNA complexed with poly-L-lysine at mass ratios of 0.4:1 and greater was retained at the gel origin. After incubation with DNase these complexes showed a high degree of stability. However, for complexes where DNA incorporation was not complete (Polypeptide:DNA = 0.2:1) free DNA was degraded as indicated by the presence of small fragments which migrated rapidly through the gel. The reduction in the intensity of the band at the gel origin also indicated the release and degradation of semi-complexed DNA.

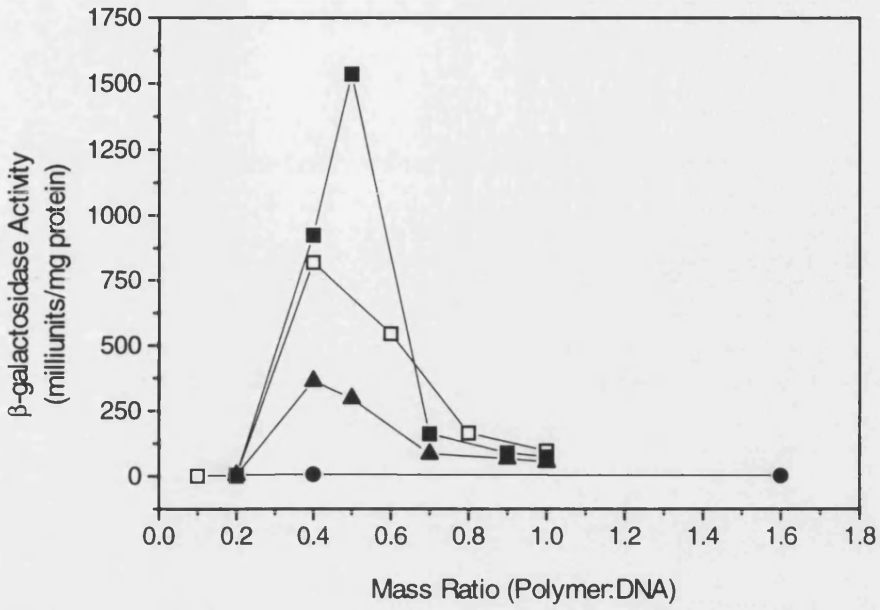


Figure 5.1. Influence of polymer-to-DNA mass ratio on gene transfer for complexes formed using poly-L-lysine of 13 (●), 127 (■), 214 (□) and 859 (▲) monomer residues. Single determinations were made in the presence of 100 μM chloroquine.

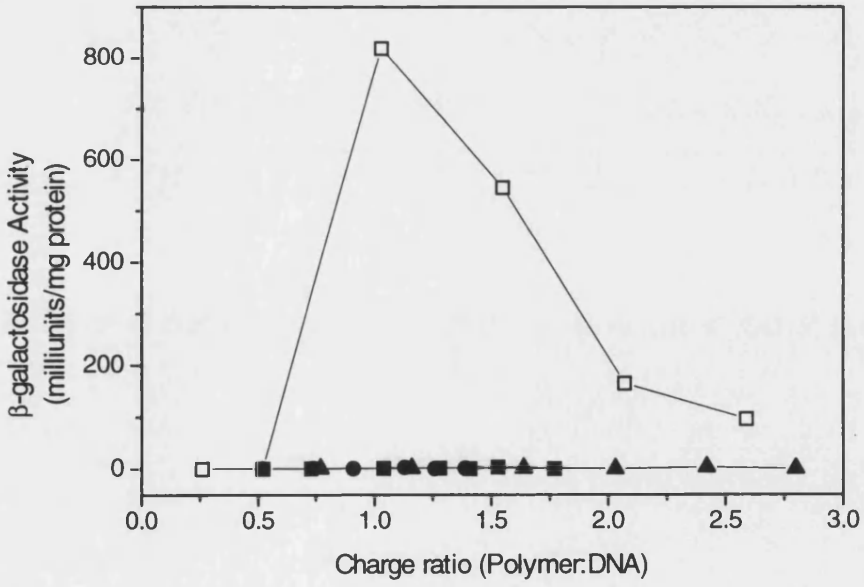


Figure 5.2. Influence of polymer-to-DNA mass ratio on gene transfer for complexes formed using AK100 (●), AK200 (■), AK500 (▲), and poly-L-lysine (□). Single determiantions were made in the presence of 100 μM chloroquine.

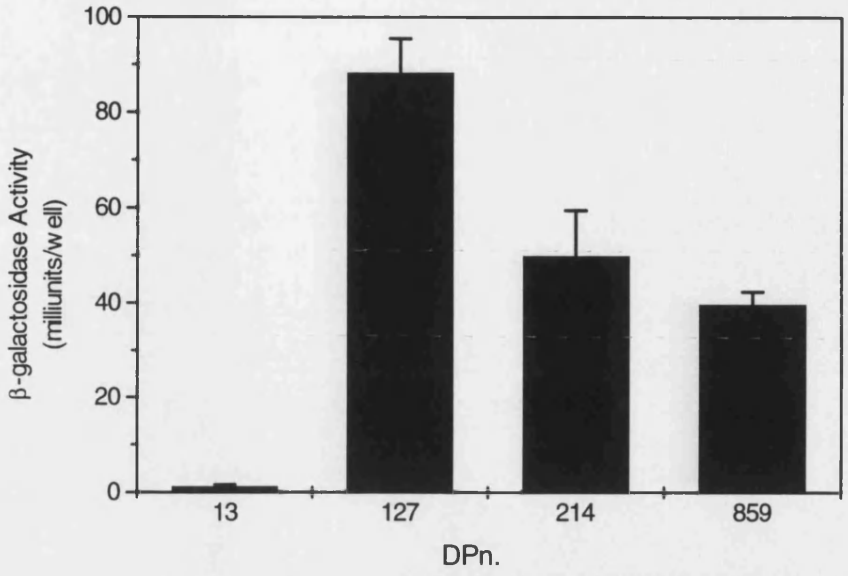


Figure 5.3. Relative levels of gene expression following transfer of pRSVlacZ to B16 melanoma cells using poly-L-lysine polymers of 13, 127, 214 and 859 monomer residues. The optimised complex formulations are described in section 5.7. Data represents the mean ± SEM for three replicate plates.

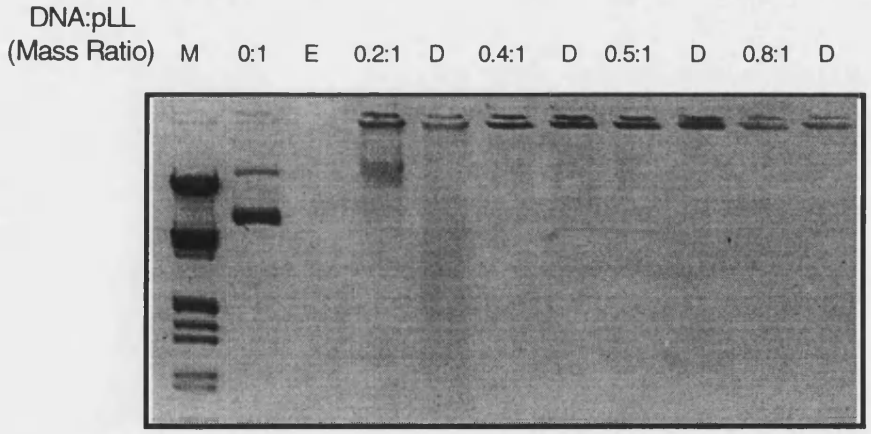


Figure 5.4. Enzymatic digestion of polylysine(214)-DNA complexes of variable polypeptide composition. Complexes containing an increasing mass of poly-L-lysine and the corresponding DNase digest (D) were analysed on a 1% agarose gel. E=empty, M=*EcoR*1/*Hind*III λDNA lane marker.

5.9. Zeta potential measurements.

The zeta potentials of poly-L-lysine-DNA complexes are described as a function of the polymer-to-DNA ratio in Figure 5.5. In preliminary experiments, measurements were made in HBS; this isotonic medium was also used in the preparation of systems for transfection. However, the high conductivity developed in this salt buffer (≈17,000 μS) resulted in a broadening of the power spectrum output and prevented accurate calculation of zeta potentials. All further experiments were, therefore, carried out in 12 μM TE buffer (pH 8.0) which eliminated peak broadening and gave sharp responses of the type shown in Figure 5.6. This medium did not affect the process of complexation (see section 2.7.2.). Under the given conditions, zeta potentials between -44 and +33 mV were obtained on modifying the polymer-to-DNA mass ratio from 0.2:1 to 5.0:1. However, charge inversion occurred rapidly between the mass ratios of 0.2:1 and 0.4:1 and from interpolation of this plot an 'isoelectric point' at a mass ratio of 0.35:1 was determined. Further increasing the polymer-to-DNA mass ratio produced complexes with higher zeta potentials. However, the relationship between zeta potential and mass ratio was not linear and at ratios of greater than 1.0:1 the charge measured on particles was relatively constant. The measured and theoretical charges of complexes are compared in Figure. 5.5.b. At the estimated point of charge neutralisation (Polymer:DNA;(+/-) = 1.0) the zeta potential of polymer-DNA complexes (+3mV) was close to zero.

5.10. Electron microscopy studies.

The shape of the poly-L-lysine (214)-DNA complexes formed at a polypeptide-to-DNA mass ratio of 0.4:1 are shown in Figure 5.7. Electron microscopy revealed a profound condensation of plasmid DNA which in most cases produced, distinct, roughly spherical particles (Fig. 5.7.a-c). At higher magnifications the polydisperse nature of the particle size distribution is apparent (Fig. 5.7.b) with condensates

between 10 and 200 nm being observed. However, the particles remained mostly discreet. Further increases in magnification (Fig. 5.7.c) failed to show any distinct sub-structure to the complexes.

Examination of complexes formed at higher polypeptide-to-DNA mass ratios indicated distinct differences in morphology. Condensates formed at a mass ratio of 0.4:1 were generally discreet and were generally less than 100 nm in diameter (Fig. 5.8.a). However, on increasing the polypeptide-to-DNA mass ratio to 1.0:1 (Fig. 5.8.b) the number of small spherical particles in the sample decreased and some aggregation became evident. Continued addition of increasing quantities of polymer (Fig. 5.8.c) resulted in significant aggregation with a distinct network of polymer evident between the particles. Some complexes also adopted a rod-like conformation. At higher magnifications these complexes exhibited a similar amorphous structure to that seen in Figure 5.7.c.

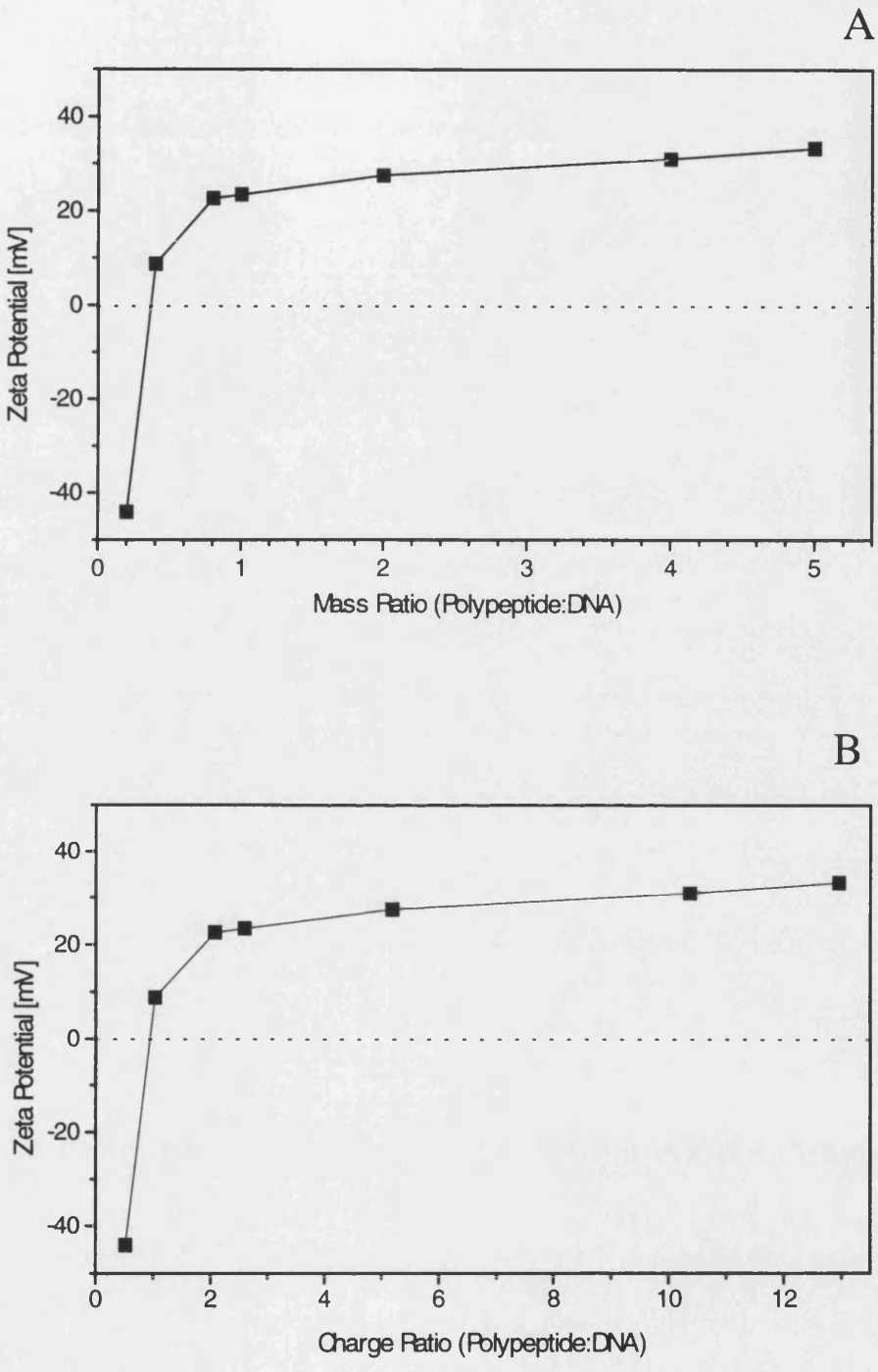


Figure. 5.5. Effect of polymer-to-DNA ratio on the zeta potential of poly-L-lysine(214)-DNA complexes in terms of mass (Plate A) and theoretical charge (Plate B). Measurements were made as described in section 5.5.

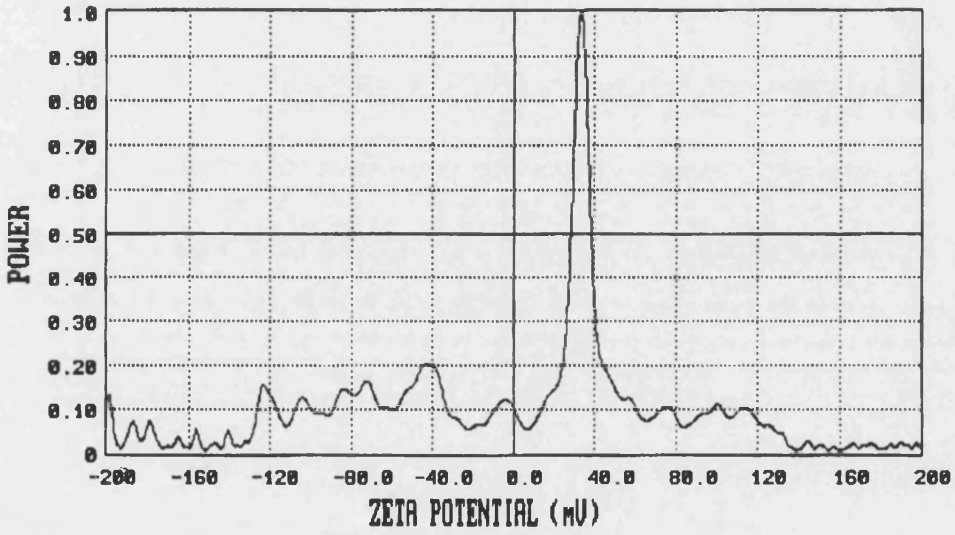
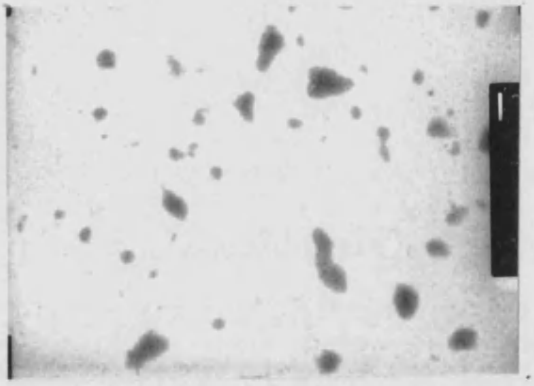
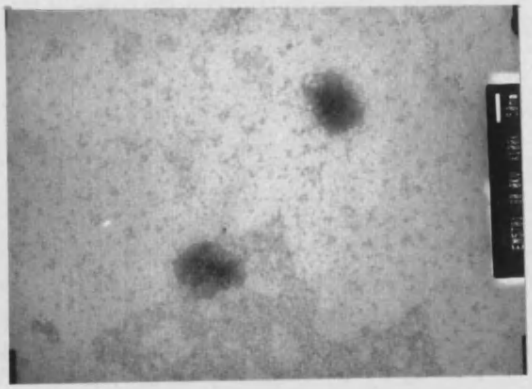


Figure 5.6. Representative ELS power spectrum for a positively charged polypeptide-DNA complex. The power spectrum was obtained for a complex formed between poly-L-lysine(214) and pRSVlacZ at a polymer-to-DNA mass ratio of 1.0:1.

(a)

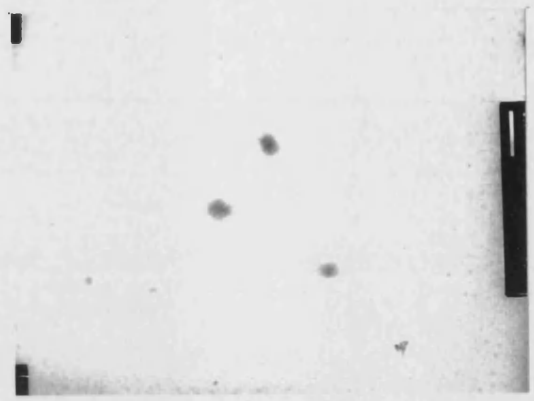


(b)

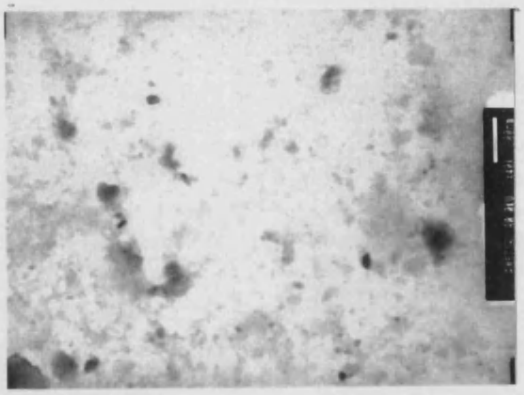


(c)

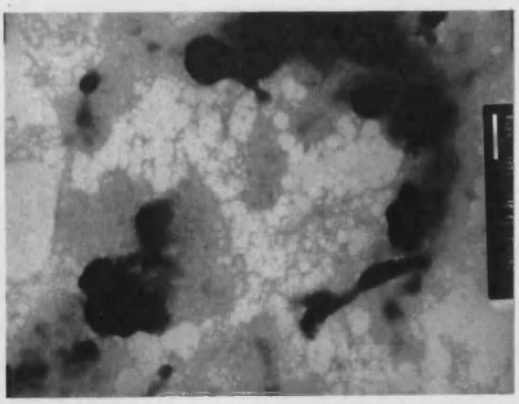
Figure 5.7. Physical characterisation of poly-L-lysine(214)-DNA complexes. Condensates prepared at a polypeptide-to-DNA of 0.4:1 were examined at magnification of 10K (a), 40K (b), and 100K (c).



(a)



(b)



(c)

Figure 5.8. Influence of polypeptide concentration on complex morphology. Condensates were prepared at a polypeptide-to-DNA mass ratio of 0.4:1 (a), 1.0:1 (b) and 5.0:1 (c). Final magnification is 40K. [Bar (applies a-c) = 200 nm].

5.11. Discussion.

5.11.1. Influence of Polypeptide-to-DNA ratio on transfection efficiency.

The efficiency with which B16 cells are transformed using polypeptide-DNA delivery systems is a function of a number of variables. In the case of streptavidin-polylysine and histone H1 complexes, a major influence was the ratio of donor DNA to polypeptide in the transfection mixture. Similar effects to those seen with streptavidin-polylysine and histone H1 were also observed for complexes formed from a series of polylysine polymers of differing chain lengths. However, using these simpler systems, it was possible to examine the underlying physicochemical changes produced on varying the polypeptide-to-DNA ratio.

5.11.2. Gene transfer mediated by electrostatic interactions.

In accordance with previous experiments, which used streptavidin-polylysine and histone H1 to facilitate gene delivery, the increase in β-galactosidase expression observed with the poly-L-lysine polymers was not gradual but was produced on achieving a specific DNA-to-polypeptide mass ratio. Calculations showed this ratio to be near or above the theoretical point of charge neutralisation suggesting the possibility that the non-specific association between polylysine-DNA complexes and the cell surface was the result of an electrostatic interaction. Zeta potential measurements made on complexes between poly-L-lysine (214) and pRSVlacZ showed the overall surface charge of particles within the system was a function of the polypeptide-to-DNA ratio. Similar observations have recently been made by Mumper *et al*, (1994) for complexes formed between polyamidoamine cascade polymers and plasmid DNA. Schreier *et al*, (1994) also showed that the addition of DOTMA/DOPE cationic liposomes to DNA results in changes in the zeta potential of the system; continued addition of liposomes produced a formulation with a net positive charge. For poly-L-lysine(214)-DNA complexes the transition from an anionic to a cationic system was produced at a polypeptide-to-DNA mass ratio

between 0.2:1 and 0.4:1. Since it was also between these ratios that complexes started to function as effective delivery systems the uptake of DNA would appear to be charge-mediated. The mechanistic implication of these data is that a sufficient excess of polymer is required to neutralise the negatively charged DNA in a polylysine-DNA complex, so that interaction with the electronegative cell surface can occur. This is in addition to the requirement for charge neutralisation to induce DNA collapse. From the enzymatic stability studies the neutralisation of charge and DNA condensation is also implicated in the protection of the nucleic acids from degradation.

As non-specific gene transfer is charge-mediated, then it may be assumed that the highest level of gene expression would be achieved with formulations having the most positive zeta potential. However, as the zeta potential of complexes with a polypeptide-to-DNA mass ratio of 1.0:1 and greater is relatively constant, an indication that negatively charged binding sites on DNA are fully occupied, then using systems containing increasing amounts of polymer would not serve to increase charge-mediated interactions. Indeed, transfection of cells with poly-L-lysine(214)-DNA complexes was optimal at a mass ratio of 0.4:1. It is therefore possible that although complexes must be cationically charged to induce DNA uptake the magnitude of this charge is not important in determining gene transfer. Alternatively, at higher polypeptide-to-DNA ratios some competing factor may be reducing gene transfer.

5.11.3. Physical characterisation of polylysine-DNA complexes.

There are relatively few structure-function studies involving the internalisation of polymer-DNA structures into eukaryotic cells. However, Wagner *et al,* (1991) advanced the theory that the biological activity of transferrin-poly-L-lysine-DNA complexes, targeted to the transferrin receptor in various cell lines, correlates to the formation of condensates of 80-100 nm in diameter. Examination of complexes

formed between poly-L-lysine(214) and pRSVlacZ, at different polypeptide-to-DNA mass ratios, showed structures with distinctly different morphology. When the polypeptide-to-DNA mass ratio was increased complexes proceeded from a discreet to an aggregated state. Since charged pharmaceutical systems are generally stable (Washington, 1990) it is likely that this aggregation results from multi-point binding between particles during the process of DNA condensation. The diameter of particles prepared at a polypeptide-to-DNA mass ratio of 0.4:1, the optimum for biological activity, was significantly smaller than the size of the aggregates seen in systems prepared with higher quantities of polymer.

It therefore seemed likely that the reproducible diminution of delivery efficiency observed with carrier formulations with high polypeptide-to-DNA mass ratios was a result of the change between small spherical complexes and aggregated systems. Bond and Wold (1987) noted a similar change in the bioactivity of poly-L-ornithine-DNA complexes but were unable to account for the phenomenon. The size and morphology of complexes therefore appears to significantly affect gene transfer efficiency and is determined by factors including the DNA-to-polypeptide ratio. However, as the size distribution of complexes within a system is polydisperse then the possibility arises that only a sub-set of the total particle population is active; smaller particles being capable of negotiating nuclear pores. The presence of some small particles in aggregated formulations may explain the low levels of gene expression detected in cells following transfection. This hypothesis is supported by Plank *et al*, (1994) who observed the slow formation of polylysine-DNA precipitates of up to 1 μM in diameter in a gene transfer system. Using density gradient centrifugation, precipitates and smaller complexes were isolated and used in a subsequent gene transfer experiment; the transfection efficiency with the soluble fraction was 20-fold higher than with the precipitate fraction. Although no direct experimental evidence is available it is possible that extensive cross linking in

polymer-DNA systems could totally prevent gene transfer. This effect may explain the inability of the alanine-lysine graft co-polymers to facilitate gene transfer despite inducing DNA condensation.

5.11.4. Transfection efficiency as a function of chain length.

Although the mass of polylysine polymer required to induce DNA condensation was previously shown to be independent of chain length (see section 2.7.) this property appeared to significantly influence the level of β-galactosidase expression following gene transfer. From these data, complexes formed with poly-L-lysine(127) were twice as efficient as those complexes produced with longer chain polymer molecules containing 214 and 859 lysine monomer units. As cross-linking has been shown to affect transfection efficiency then it is feasible that the increased gene expression seen with polylysine(127) resulted as complexes formed with this shorter polypeptide were less likely to aggregate. The very short chain length polymers, represented by polylysine(13), did not induce effective DNA condensation and were unable to facilitate gene transfer at any polypeptide-to-DNA ratio tested. Therefore, in optimising the length of the cationic carrier for gene transfer systems it would appear that a compromise must be achieved between DNA condensation and cross-linking.

5.12. Summary.

The data presented in this chapter shows that only positively charged polypeptide-DNA complexes are taken up by B16 melanoma cells. The non-specific association between polylysine-DNA complexes and the cell surface was therefore on an electrostatic basis. However, the morphology of complexes also determines the efficiency of gene transfer; it is probable that smaller particles are more bioactive. In addition, it appears that the chain length of polypeptides used to form complexes affects the efficiency of gene transfer.

Chapter 6

Strategies for targeting gene delivery to B16 melanoma cells.

Strategies which circumvent problems with non-specific gene transfer generally involve delivering DNA to cells by receptor-mediated endocytosis. The mechanism proposed in this study to achieve selective gene delivery to melanoma cells is to use a derivative of [Nle^4,D-Phe^7]α-MSH as a targeting agent. This peptide has a high affinity at the murine MC1 receptor (Sawyer *et al*, 1980). Conjugation to the vector was by the polylysine-streptavidin-biotin link described in chapter 3. However, for efficient delivery of DNA by receptor-mediated endocytosis the conjugate must be rapidly and specifically taken up by the target cell; this requires a high affinity of the ligand for the receptor, high receptor density and a high rate of endocytosis/internalisation. Consequently, the initial focus of the following experiments was to ensure N$^{\alpha}$-biotin-(Gly$_3$)-[Nle^4,D-Phe^7]α-MSH retained the ability to specifically bind at the MC1 receptor on conjugation to the outer coat of the vector. A second targeting strategy which utilises the binding of lectins to cell surface glycoconjugates was also tested. These molecules are overexpressed on neoplastic cells (Hynes, 1976; Nicolson, 1982).

6.1. Peptides.

Peptides were prepared in our laboratory using solid phase synthesis by Dr G.W.J. Olivier, and their preparation is therefore not described in detail. Lyophilised peptides were reconstituted with sterile 0.1 mM HCl and stored at 4°C.

6.1.1. Biotinylation of N$^{\alpha}$-(Gly$_3$)-*[Nle^4,D-Phe^7]*α-MSH.

Biotinylation of peptides was demonstrated by qualitative detection with avidin conjugated horseradish peroxidase (Sigma). Samples containing 1 mg/ml N$^{\alpha}$-biotin-

(Gly_3)-[Nle^4,D-Phe^7]α-MSH were added, dropwise, onto a nitrocellulose membrane (0.45 μM pore size). An equal mass of N^{α}-(Gly_3)-[Nle^4,D-Phe^7]α-MSH was blotted on the same membrane as a control. After air drying at ambient temperature, non-specific binding was blocked using the method previously described for affinity blotting (section 3.8.2.). Following this, membranes were rinsed with TBS before incubating with avidin-HRP (2 μg/ml), for 60 minutes. Blots were developed as before; a positive purple colour reaction showed the test peptide had been biotinylated.

6.2. Radioiodination of α-MSH analogues.

Radioactive iodine was coupled at the Tyr^2 position of the α-MSH analogues, [Nle^4,D-Phe^7]α-MSH and N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH, by the oxidative chloramine T method described by Eberle and Hübscher, (1979).

6.2.1. Column chromatography solutions.

1% v/v TFA.
50%, 60% and 80% v/v methanol +1% v/v TFA.
0.25 M sodium phosphate buffer, pH 7.4.

6.2.2. Iodination reaction reagents.

0.25% w/v BSA in 0.05 M phosphate buffer pH 7.4.
1% w/v Polypep (Sigma) in 0.05 M phosphate buffer, pH 7.4.
0.1% w/v chloramine-T in distilled water (prepared immediately before use).

6.2.3. Preconditioning of purification columns.

A C18 reverse phase column packed with Spherisorb ODS (Anachem), was preconditioned by washing according to the following protocol:

3 x 1 ml 1% v/v TFA.
3 x 1 ml 80% v/v methanol/ 1%TFA.
1 x 1 ml 1% w/v Polypep solution (protein digest).
3 x 1 ml 1% v/v TFA.

6.2.4. Peptide iodination.

Prior to the iodination reaction, 20 μg [Nle^4,D-Phe^7]α-MSH or N$^\alpha$-biotin-(Gly$_3$)-[Nle^4,D-Phe^7]α-MSH was diluted to 20 μl in 0.25 M phosphate buffer (pH 7.4). To the peptide solution 10 μl Na^{125}I (nominally 37 Mbq activity) was added, and the oxidation reaction then initiated using 10 μl 0.1% ^{w}/$_{v}$ chloramine-T solution. After 30 seconds the reaction was quenched with 0.6 ml 0.25% ^{w}/$_{v}$ BSA. To remove unreacted Na^{125}I the reaction mixture was applied to a preconditioned ODS column which was then washed twice with phosphate buffer. The iodinated ligand was eluted by washing the column with 4 x 1 ml 50% ^{v}/$_{v}$ methanol/1%^{v}/$_{v}$ TFA followed by 2 x 1 ml 60% Methanol/1% TFA. The eluate was retained for further purification by reverse phase HPLC.

6.2.5. Purification of radiolabelled peptides.

Eluate from the iodination reaction contained a mixture of mono- and di-iodinated peptides necessitating further purification by HPLC. An exponential gradient of 0.1% TFA in water and 0.1% TFA in 70% acetonitrile/30% water was used with fractions collected at one minute intervals from 25-40 minutes after injection. The mono-iodinated compound [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH, which was used as a radiotracer in competitive binding experiments, eluted 35 minutes after injection; with the di-iodinated peptide eluting approximately three minutes later. N$^\alpha$-biotin-(Gly$_3$)-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH was isolated using the same chromatographic conditions and was found to elute 36 minutes after injection. The di-iodinated peptide eluted after 39 minutes. Radioactivity in each fraction was quantified on a LKB 1277 Gammamaster gamma counter. Those fractions associated with the mono-iodinated peptide peak were pooled, their collective activity measured as counts per minute (cpm), and the radiotracer concentration determined as outlined in section 6.2.6.

6.2.6. Calculation of radiotracer concentration.

The concentration of radiotracer was calculated from the measured radioactivity by the following method:

By definition, [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH contains 1 mole of ^{125}I per mole of peptide.

$\therefore$ 1 matom ^{125}I $\equiv$ 1 x 10^{-3} moles [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH.

As carrier free Na^{125}I (Amersham) has a specific activity of 80.5 x 10^{12} Bq/matom then 1 mole of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH has an activity of 80.5 x 10^{15} Bq.

1 Bq = 1 decay per second or 60 decays per minute and the efficiency of the gamma counter = 70%.

$\therefore$ 1 mole [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH would register 80.5 x 10^{12} x 10^3 x 60 x 0.7 cpm = 3.38 x 10^{18} cpm on the LKB 1277 Gammamaster gamma counter.

This relationship was also used to determine the concentration of N$^{\alpha}$-biotin-(Gly$_3$)-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH.

6.3. Formation of streptavidin-peptide conjugates.

Prior to use streptavidin (Sigma) and FITC-streptavidin (Sigma) were dissolved in PBS at a concentration of 3.3 μM and stored in 0.5 ml aliquots at 4°C. Immediately before use, N$^{\alpha}$-biotin-(Gly$_3$)-[Nle^4,D-Phe^7]α-MSH (1 mg/ml) was diluted in PBS to a concentration of 3.3 μM. For quantitative assessment of conjugate formation the peptide reaction solution was spiked with 5 μl of a 2.4 x 10^{-8}M solution of N$^{\alpha}$-biotin-(Gly$_3$)-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH (molar ratio labelled : unlabelled peptide = 1 : 14,000). Conjugates were then prepared by adding 0.5 ml of peptide

solution to an equal volume of streptavidin or FITC-streptavidin solution. The reaction mixture was then incubated with constant agitation at 4°C for 60 minutes.

6.3.1. Purification of streptavidin-peptide conjugates.

Uncomplexed biotinylated peptide was removed from reaction mixtures using streptavidin coated magnetizable beads (Dynabeads M-280, Dynal). These beads are non-porous, uniform, supermagnetic polystyrene particles. Prior to use, beads were washed twice using 0.1% BSA/PBS in order to remove sodium azide and reduce non-specific binding. The beads were then resuspended at 10 mg/ml in 0.1% BSA/PBS. All manipulations were conducted under aseptic conditions using filter-sterilised solutions. Conjugate reaction mixtures were then added to 150 µl of bead suspension in a 1.8 ml screw capped microcentrifuge tube and the suspension incubated, with gentle agitation, at 4°C for 60 minutes. Following this, the tube was placed in a strong magnetic field (Magnetic Particle Concentrator; Dynal) for five minutes. During this time the magnetic beads with bound biotinylated peptide were attracted to the wall of the tube, forming a firm pellet. The supernatant containing the peptide-streptavidin complexes was then carefully removed using a pipette while the magnet remained in place.

6.3.2. Validation of purification.

The purification procedure was validated using a reaction mixture spiked with the radiolabelled peptide N^{α}-biotin-(Gly_3)-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH. Conjugates were prepared as described in section 6.3.1. Following purification of the reaction mixture, the supernatant was aspirated and retained. The beads were then released from the magnet and washed with 2 x 1.0 ml aliquots of PBS. Magnetic separation was performed between each washing. Finally, the beads were resuspended in 0.5 ml PBS. Radioactivity remaining in the supernatant and on the beads was measured using a gamma counter. The proportion of ^{125}I activity retained on the beads was expressed as a percentage of the input. The relationship between measured

radioactivity (cpm) and the specific activity of $Na^{125}I$ was then used to calculate the total molar quantity of peptide retained on the beads or in the supernatant.

6.3.3. Specific binding of N^{α}-biotin-*(*Gly_3*)-[*Nle^4*,D-*Phe^7*]*α-MSH *by streptavidin coated magnetic beads.*

The total capacity of streptavidin coated magnetic beads to bind N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH was determined using the radiotracer described in section 6.2.5. Peptide solutions containing 1.65 nanomoles N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α -MSH spiked with the radiotracer were added to 150 µl of bead suspension (1.5 mg) and incubated for 60 minutes at 4°C. Radioactivity retained on the beads was then measured. The contribution of non-specific binding was determined by blocking specific binding sites by pre-incubating beads with a 1.0 ml volume of biotin solution (6×10^{-5} M) for 60 minutes at room temperature. The biotin solution was then removed, beads washed with PBS and pelleted by centrifugation at 13,000 rpm for 5 seconds. Beads were resuspended in 150 µl PBS and used immediately.

6.3.4. 2-iminobiotin chromatography.

Formation of conjugates between streptavidin and N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7] α-MSH was confirmed using 2-iminobiotin affinity chromatography. A column was prepared from a 1 ml disposable plastic syringe and packed with a 2-iminobiotin modified 6% beaded agarose gel (Sigma) to give a settled volume of 0.25 ml. The gel matrix was supported by a porous PTFE disk inserted at the bottom of the syringe barrel. Purified FITC-streptavidin-N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH conjugate was then applied to the column which had been previously been equilibrated with 10 column volumes (2.5 ml) of binding buffer (50 mM sodium carbonate, 1 M sodium chloride). Conjugates used in this experiment contained the radiolabelled peptide N^{α} -biotin-(Gly_3)-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH. The column was washed with 0.5 ml aliquots of phosphate buffered saline (pH 7.3) until background levels of ^{125}I activity

were observed in the collected fractions. Following this, the wash buffer was changed to 0.1 M acetic acid and ^{125}I activity in the eluted fractions measured.

6.4. Binding of peptide-conjugates at the MC1 receptor.

Competitive binding assays were carried out following a method adapted from Siegrist *et al*, (1988) as described by Erskine Grout (1993).

6.4.1. Binding medium.

RPMI 1640 medium containing 25 mM Hepes and 0.2% BSA was prepared immediately before use. Hepes solution containing BSA was prepared as a 10X concentrate in serum free RPMI 1640 and stored at -20°C until used.

6.4.2. Competition Binding.

B16 cells were detached from flasks, counted and seeded at a density of 5 x 10^5 cells per well into 24 well plates and incubated for 15 hours under standard conditions. The medium was aspirated from the wells and the cells washed twice with ice-cold RPMI 1640 without additives and placed on ice to cool to 0-4°C. A fixed concentration of the radiotracer [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH, nominally 0.1 nM, and serial dilutions of the conjugate or test peptide were added to the binding medium. Concentrations in the range of 1 x10^{-6} to 1 x 10^{-12} M were used. Following this, 0.5 ml of the binding medium containing the test compound and the radiotracer was added into each well. After an 8 hour incubation at 0-4°C, unbound radiotracer was removed and the cells washed twice with RPMI 1640 medium without additives. The cells were then lysed with 1 M NaOH and the residual radioactivity counted.

6.4.3. Analysis of binding data.

MINSQ non-linear least square regression analysis was used to calculate dissociation constants from the binding data employing the following equation:

$$\text{cpm(test)} = \frac{(\text{cpm}_{(\text{max})} - \text{cpm})_{(\text{min})} \times [\text{A}]}{[\text{A}] + K_{da} \times \left(\frac{[\text{B}]}{K_{db}} \right)} + \text{cpm}_{(\text{min})}$$

with cpm(test) = cpm for sample point

cpm_{max} = maximum bound (no competitor)

cpm_{min} = minimum bound (excess competitor)

[A] = Concentration of radiotracer

[B] = Concentration of competitor

K_{da} = dissociation constant of [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH (0.48 nM)

K_{db} = dissociation constant of competitor

6.5. FITC-Concanavalin A binding to B16 melanoma cells.

Labelling of unfixed B16 melanoma cells was carried out with concanavalin A which was derivatised with fluorescein-isothiocyanate (Sigma). Washing and labelling procedures were conducted at 4°C to avoid endocytic uptake of lectins. Cells were harvested from confluent cultures using 0.02% EDTA/PBS, washed twice with PBS, and resuspended at 1 x 10^6 cells/ml. To 0.5 ml of cell suspension an equal volume of labelling solution containing FITC-Con A in PBS was added and the mixture incubated for 90 minutes with occasional agitation. FITC-Con A was used at a final concentrations of 12.5 µg/ml. The specificity of lectin binding to the cells was established by incubating cells with FITC-Con A in the presence of 0.1 M maltose. To eliminate the possibility of fluorescent changes resulting from

differences in osmolarity, control samples were also prepared in the presence and absence of the specific inhibitory sugar. After incubation, cells were washed twice in ice cold PBS, pelleted by centrifugation, and resuspended in 1.0 ml of PBS. Cell fluorescence was analysed by flow cytometry.

6.6. Formation of binary complexes for gene targeting studies.

Prior to the construction of tertiary complexes containing ligands for gene targeting, binary complexes of plasmid DNA and streptavidin-poly-L-lysine were pre-formed. Complexes were prepared as follows; 6.0 μg pRSVlacZ in 250 μl HBS was mixed with an equal volume of HBS containing 2.6 μg streptavidin-polylysine(219) and incubated at room temperature for 30 minutes. The formulation chosen (Polypeptide:DNA;(μg:μg) = 0.43:1) ensured complete DNA complexation but minimised non-specific gene transfer.

6.6.1. Preparation of vectors containing N^{α}-biotin-*(Gly$_3$)-[Nle^4,D-Phe^7]*α-MSH.

Vectors containing N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH were derived by varying the amount of biotinylated-peptide added to pre-formed binary complexes. A 1 mg/ml stock solution of N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH was diluted to 2 μg/ml in HBS and aliquots added to conjugate suspensions to give complexes. Peptide containing conjugates were incubated for a further 30 minutes before use. Complexes used in the experiments contained either 45 or 22.5 molecules per complex. The ratio of peptide ligand-to-streptavidin in the complexes was 1:1 and 0.5:1 respectively. Hence, these complexes were designated as 1.0 X and 0.5 X conjugates.

6.6.2. Preparation of vectors containing concanavalin A.

Targetable concanavalin A vectors were prepared from the binary complexes described in section 6.6. Biotinylated ConA (Sigma) was dissolved at a

concentration of 20 nmoles/ml in filter sterilised HBS according to the manufacturer's instructions and aliquots added to conjugate suspensions to give between 4.5 and 45 ConA molecules per complex. These complexes were designated as 0.1 X and 1.0 X conjugates respectively. Systems were treated as described in section 6.6.1.

RESULTS

6.7. Preparation of N^{α}-biotin-(Gly_3)-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH.

The conjugation of streptavidin and biotinylated peptides was followed using a radiotracer prepared by iodination of N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH. The mono-iodinated form of this peptide was isolated by reverse phase HPLC (Fig. 6.1.).

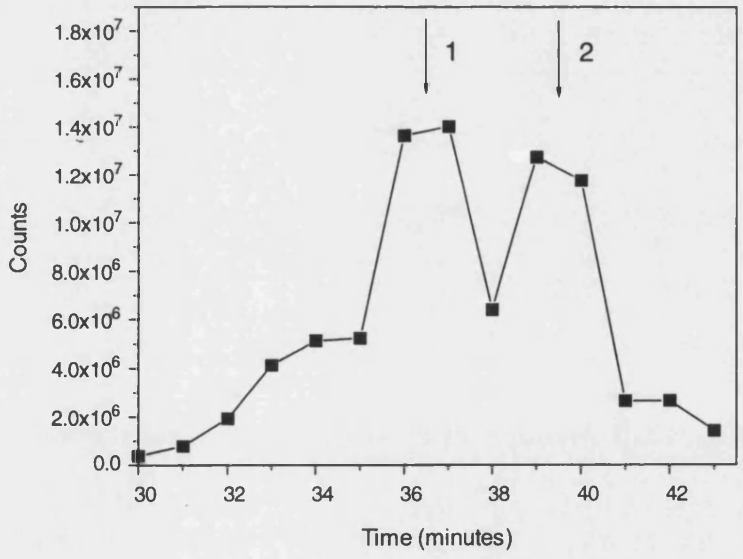


Figure 6.1. Elution profile of mono- and di-iodinated N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH from analytical scale HPLC. N^{α}-biotin-(Gly_3)-[^{125}I-Tyr^2,Nle^4,D-Phe^7] α-MSH (1) eluted at 36 minutes and N^{α}-biotin-(Gly_3)-[$(^{125}I)_2$-Tyr^2,Nle^4,D-Phe^7]α-MSH (2) at 39 min.

6.8. Confirmation of conjugate formation using 2-iminobiotin chromatography.

In preliminary experiments the peptides produced by solid phase synthesis were shown to be biotinylated by affinity blotting with avidin-HRP. However in order to validate the binding of these peptides to streptavidin, purified reaction mixtures were qualitatively analysed by 2-iminobiotin affinity chromatography. As can be seen from Figure 6.2., the radiolabelled peptide N^{α}-biotin-(Gly_3)-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α -MSH, which had been incubated with FITC-streptavidin, was extensively retained on the column. A small quantity of ^{125}I activity was eluted on loading although washing the column with phosphate buffered saline (pH 7.3) rapidly reduced radioactivity in the collected fractions to background levels. Complexes containing the iodinated radiotracer were then released from the column by washing with low pH buffer (0.1 M acetic acid). The majority of applied ^{125}I activity (60%) was found in these specifically eluted fractions.

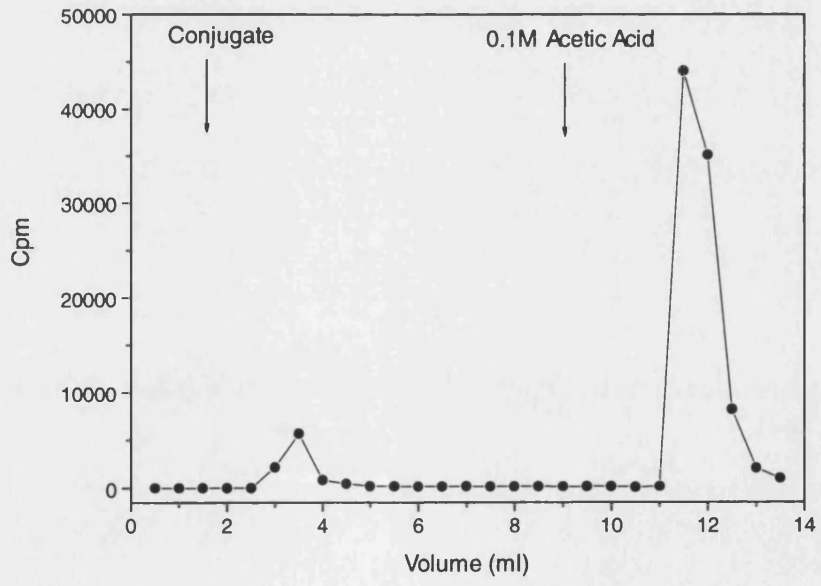


Figure 6.2. Elution of FITC-streptavidin-N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH complexes from a 2-iminobiotin affinity column. Complexes containing the radiotracer N^{α}-biotin-(Gly_3)-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH were loaded on to the column at pH 11.0 and specifically eluted by application of 0.1 M acetic acid.

6.9. Validation of purification.

The results presented in Table 6.1. show the capability of the streptavidin coated magnetic beads to specifically bind N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH. Incubation of 1.5 mg of beads with peptide solution resulted in the retention of 46.5 ± 6.0% of the applied radioactivity. The contribution of non-specific effects to this total was assessed by blocking binding sites on the bead surface. Under these conditions ^{125}I activity associated with the beads was reduced to 3.7 ± 0.5% of the applied dose. At the end of the 60 minute incubation period a concomitant increase in ^{125}I activity remaining in the supernatant (83.7 ± 1.1%) was also demonstrated. Using these data it was calculated that each milligram of bead suspension binds a maximum 442 picomoles of N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH. Conjugation of streptavidin and N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH was followed by measuring the relative amounts of activity remaining in the reaction supernatant or bound onto the beads. The quantity of N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH removed following purification of streptavidin-peptide reaction mixtures (58 ± 25 pmols; n=3) was significantly less than the calculated maximum capacity. The majority of the radiolabel (94.4 ± 1.4%;n=3) was held in solution indicating successful complexation. In an attempt to establish if any biotinylated peptide remained in solution at the end of the incubation period ^{125}I activity was measured on the purification beads and on beads pre-incubated with biotin. The amount of peptide retained was not significantly different under each of the conditions ($p > 0.05$, *t* test unpaired). Similar results were obtained with FITC-streptavidin.

6.10. Binding at the MC1 receptor.

Dissociation constants of N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH and conjugates with streptavidin and FITC-streptavidin at the murine MC1 receptor were estimated from the radioactivity associated with the cells as described in section 6.4. The means and standard deviations of three or more experiments were determined for each compound. Incubation of cells with [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH in the

Table 6.1. Retention of N^{α}-biotin-(Gly_3)-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH by streptavidin coated beads. Data is expressed as the percentage of input radioactivity retained on beads or in the supernatant (± SD; n=3) .

Sample	Supernatant (%)	Beads (%)	Balance (%)
N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH	44.1 3.7	46.5±6.0	90.5±2.3
N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH (Beads biotin blocked)	83.7±1.1	3.7±0.5	87.6±1.1
Streptavidin-N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH	94.4±1.6	3.5±1.5	97.8±0.5
Streptavidin-N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH (Beads biotin blocked)	89.5±1.1	4.4±0.9	93.9±0.3
FITC-Streptavidin-N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH	91.8±0.4	5.4±0.4	97.2±0.1

Table 6.2. Retention of N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH by streptavidin coated beads. The total quantity of peptide retained was calculated using data derived using N^{α}-biotin-(Gly_3)-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH (Table 6.1.). Data is expressed as the number of picomoles retained (± SD; n=3).

Sample	Supernatant (pmols)	Beads (pmols)	Total (pmols)
N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH	728±61	769±99	1497±275
N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH (Beads biotin blocked)	1381±22	64±8.5	1441±16.0
Streptavidin-N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH	1558±26	58±25	1616±6.9
FITC-Streptavidin-N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH	1515±7	89±7	1604±2.0

presence of increasing concentrations of N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH and streptavidin or FITC-streptavidin peptide-conjugates showed a dose dependent and saturable displacement of radiotracer binding (Fig. 6.3.) The dissociation constants derived by linear least square regression from the competition binding curves are given in Table 6.3. Conjugation of N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH with streptavidin or FITC-streptavidin produced species with significantly lower binding than the native peptide ($p < 0.05$, *t* test unpaired). The binding affinity of streptavidin-N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH was 40-fold lower than the native peptide. Binding affinities of conjugates formed with streptavidin or FITC-streptavidin were not significantly different ($p > 0.05$, *t* test unpaired).

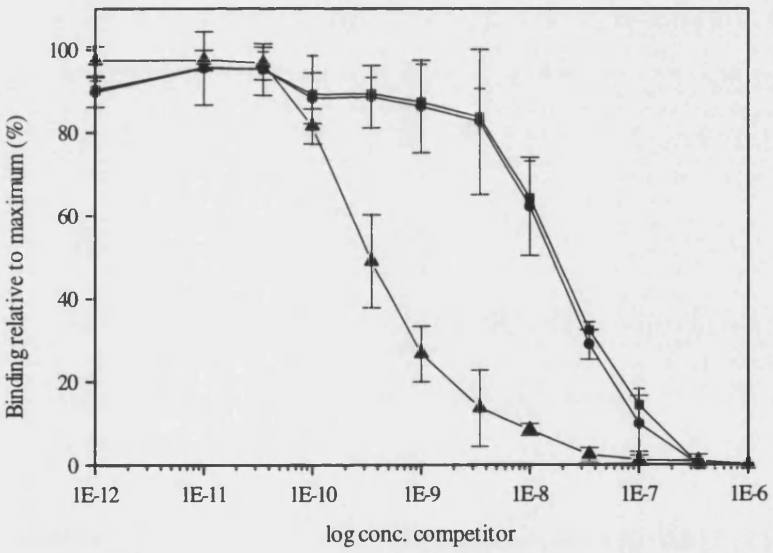


Figure 6.3. Binding activity of the biotinylated peptide and streptavidin/streptavidin-FITC peptide conjugates to the murine MC1 receptor. Confluent monolayers of cells were incubated with [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH in the presence of increasing concentrations of native N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH (▲), streptavidin-peptide complexes (●), or FITC-streptavidin-peptide complexes (■). After 8 hours at 4°C binding was terminated and the remaining ^{125}I activity measured. Data represent the mean ± SD for three replicate experiments.

Table 6.3. Dissociation constants of N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH and streptavidin-peptide complexes. Mean values of the three individual experiments are also shown.

Compound	Binding (K_{db})	Relative Binding
N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH	(1) 8.22×10^{-10} (2) 1.53×10^{-9} (3) 3.38×10^{-9} Mean = 1.9×10^{-9} S.D. = $\pm 1.3 \times 10^{-9}$	1.000
Streptavidin-N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH	(1) 7.62×10^{-8} (2) 4.19×10^{-8} (3) 1.07×10^{-7} Mean = 7.5×10^{-8} S.D. = $\pm 3.3 \times 10^{-9}$	0.025
Streptavidin-FITC-N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH	(1) 7.82×10^{-8} (2) 5.77×10^{-8} (3) 1.07×10^{-7} Mean = 8.1×10^{-8} S.D. = $\pm 2.5 \times 10^{-8}$	0.023

6.11. Gene delivery to B16 cells using N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH.

Experiments examining the possibility of delivering polylysine-pRSVlacZ conjugates via the MC1 receptor utilised conjugates containing N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH. B16 cells were incubated with complexes formulated to reduce charge-mediated uptake (Polypeptide:DNA;(μg:μg) = 0.43:1) in the presence of 100 μM chloroquine for 4 hours. As controls, cells were incubated with HBS and the streptavidin-polylysine formulation optimised for non-specific gene transfer (Polypeptide:DNA;(μg:μg) = 0.63:1). The results presented in Figure 6.4. indicate significant β-galactosidase expression following delivery of pRSVlacZ complexed with streptavidin-polylysine at polypeptide-to-DNA mass ratios of 0.43:1 and 0.63:1. However, at the higher mass ratio the level of relative enzyme activity was 4-fold greater than with the 0.43:1 formulation. The inclusion of varying quantities of N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH into complexes produced no significant increase in β-galactosidase activity compared to the control system ($p > 0.05$, *t* test unpaired). Indeed, with the 0.5 X conjugate a decrease in β-galactosidase expression was seen.

6.12. Binding of Concanavalin A to B16 melanoma cells.

Expression of concanavalin A binding sites on B16 cells was determined by flow cytometry. The frequency and fluorescence profiles of control and stained cells are presented in Figure 6.5. These results indicate that relative to the fluorescence histogram pattern of the control cell population (mean = 5.7), that of B16 cells incubated with FITC-Con A was displaced to the right (mean = 804.0). Fluorescence staining under these conditions was intense; a similar level of staining and degree of shift was also observed when cells were incubated with higher concentrations of FITC-ConA (25 and 100 μg/ml). In order to demonstrate the specificity of binding, cells were incubated with FITC-ConA in the presence of 0.1 M maltose. Under these conditions, displacement of the histogram pattern was not as great. The mean fluorescence intensity of this cell population (Mean = 147.1) was reduced to 18% of

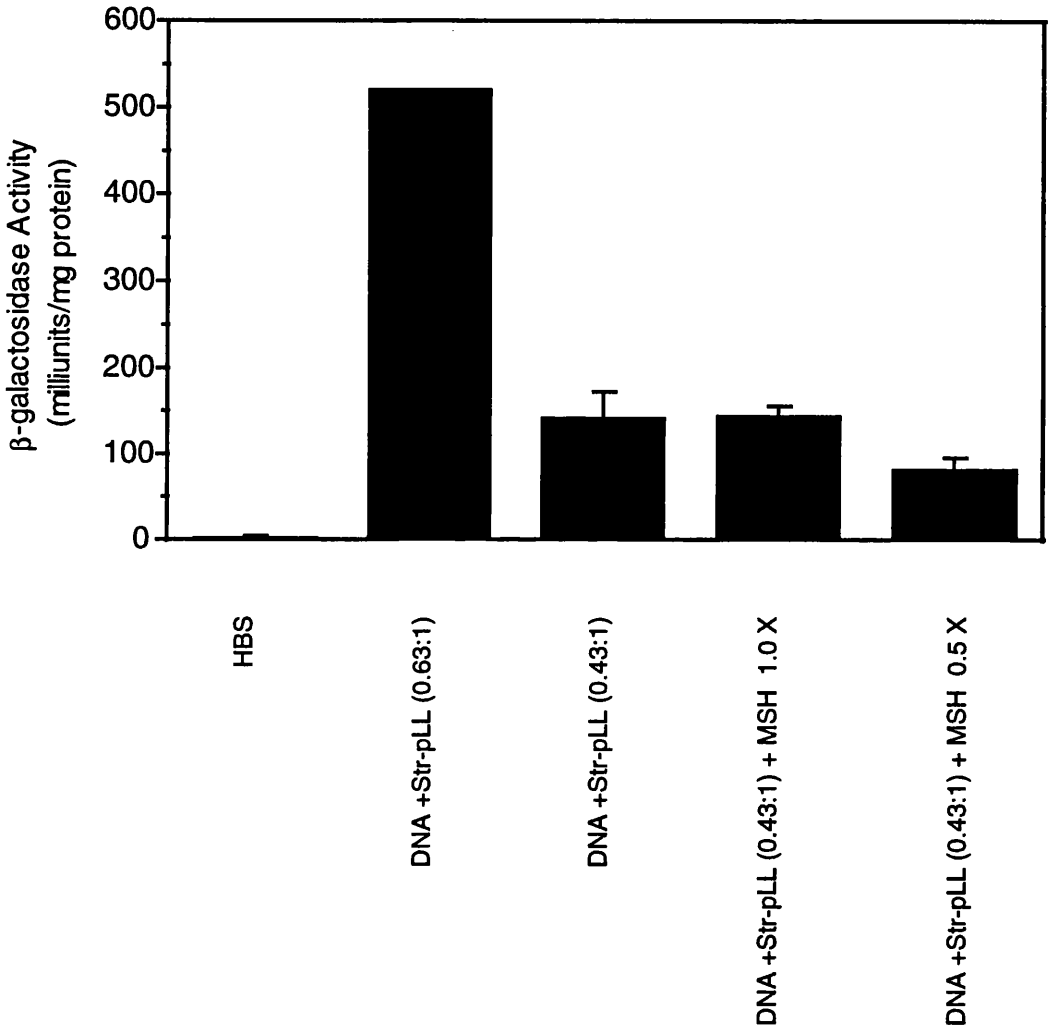


Figure 6.4. Gene transfer mediated by MSH-targeted molecular conjugates. B16 melanoma cells were transfected with molecular conjugates contained variable amounts of N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH. The 1.0 X conjugates contained 45 molecules of peptide per conjugate. Data represents the mean ± SEM for triplicate transfection.

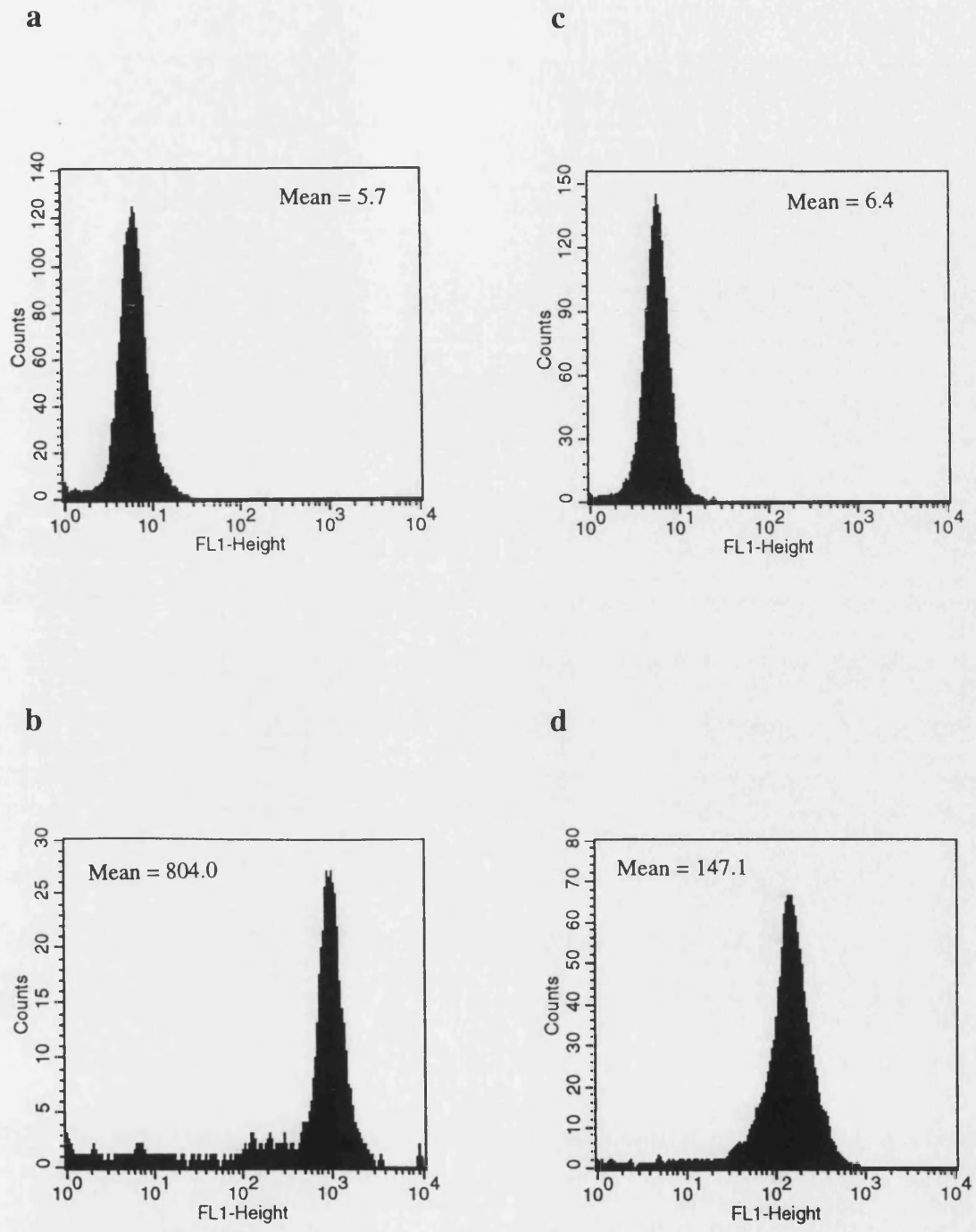


Figure 6.5. Fluorescence distribution of B16 melanoma cells stained with FITC-ConA. **Panel a**, Control; **Panel b**, FITC-ConA (12.5 µg/ml); **Panel c**, Control + 0.1 M maltose; **Panel d**, FITC-ConA (12.5 µg/ml) + 0.1 M maltose. FL1=fluorescence intensity of channel 1, green fluorescence.

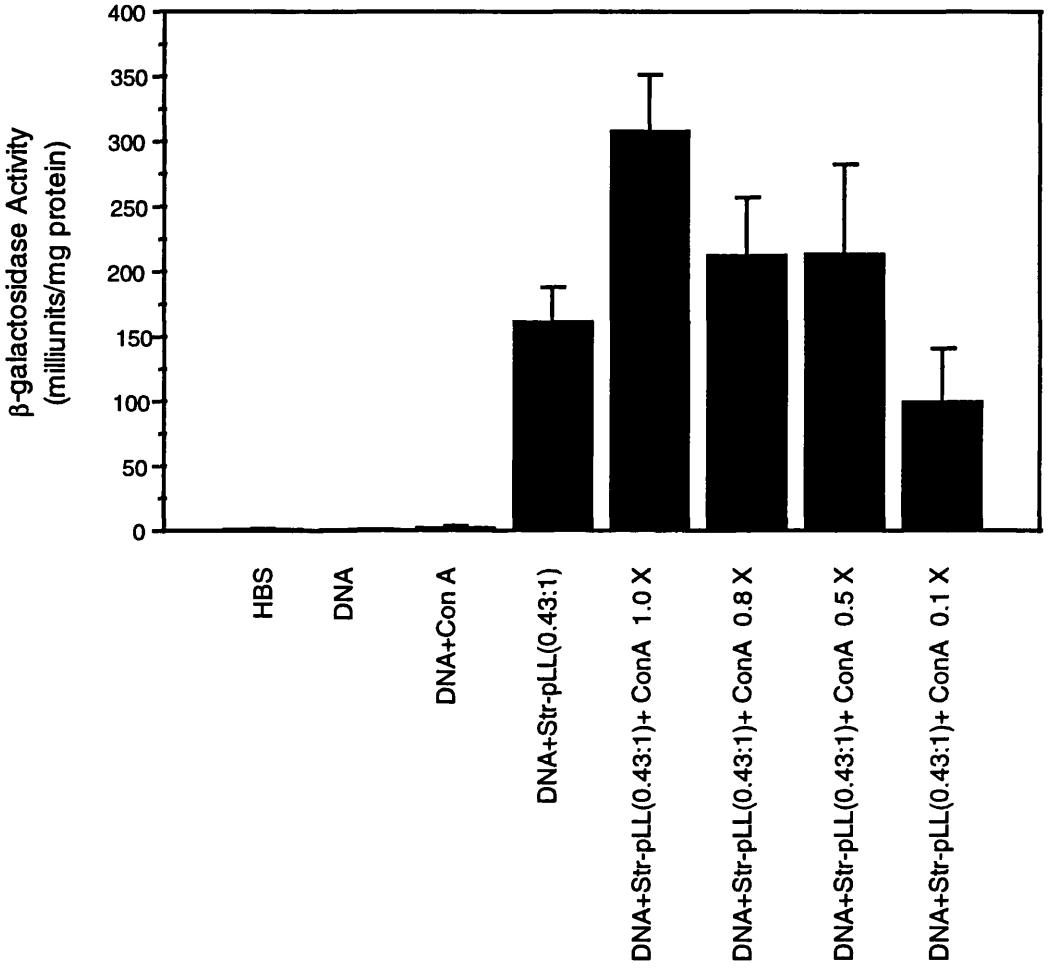


Figure 6.6. Gene transfer mediated by ConA-targeted molecular conjugates. B16 melanoma cells were transfected with molecular conjugates containing variable amounts of Con A. Data represents the mean of six replicate transfections (± SEM).

the value found following incubation of cells with FITC-ConA alone. In each case, the fluorescence histogram pattern of stained cells was similar in shape to that of the background. Cultures incubated with 0.1 M maltose (mean = 5.7) were indistinguishable from experimental cultures (mean = 6.4).

6.13. Gene delivery to B16 melanoma cells mediated by Concanavalin A.

To test the feasibility of receptor mediated gene delivery to B16 cells via lectins, cells were incubated with poly-L-lysine-DNA-Con A complexes for 4 hours in the presence of 100 μM chloroquine. As controls, cells were also treated with blank medium (HBS), pRSVlacZ (6 μg) and pRSVlacZ in combination with Con A (200 nanomoles/ml). The results, shown in Figure 6.6. indicate that neither DNA or DNA+Con A produced high levels of gene transfer. In each case reporter gene expression was not significantly different from background levels ($p > 0.05$, *t* test unpaired). However, incorporation of varying amounts of Con A into the complex formulation produced a significant increase in gene expression ($p < 0.05$, *t* test unpaired) at a ratio of biotinylated lectin to streptavidin of 1:1 (i.e. 45 pmol biotinylated ConA: 45 pmol streptavidin).

6.14. Discussion.

Although, α-melanocyte stimulating hormone and its derivatives have been employed in targeting small molecule drugs (Varga and Asato, 1977; Lejeune and Ghanem, 1993) the majority of ligands covalently linked to polypeptide-DNA delivery systems have been globular proteins. It was therefore necessary to confirm that the proposed ligand, N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH, retained the ability to bind at the MC1 receptor after conjugation to the outer coat of the molecular vector. Evidence for binding following conjugation to streptavidin, was obtained from competitive binding assays. However, it was of concern that the efficiency with which the streptavidin-ligand complex formed was such that free peptide could remain in solution and influence any estimate of binding affinity.

6.14.1. Binding of N^{α}-biotin-*(Gly_3)-[Nle^4,D-Phe^7]*α-MSH *and streptavidin.*

Qualitative evidence for the specific binding of N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH and streptavidin was determined using 2-iminobiotin affinity chromatography. This technique is based on the work of Green, (1966), who showed that the iminobiotin-avidin dissociation constant was pH dependent and identified the free base of iminobiotin as the species that binds to avidin. These fundamental observations were applied by Hofmannn *et al*, (1980) to the chromatographic purification of streptavidin and by Fudem-Goldin and Orr, (1990) in the isolation of rhodamine-avidin conjugates. In order that the chromatographic behaviour of complexes containing N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH could be followed the radiolabelled tracer N^{α}-biotin-(Gly_3)-[^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH was included into reaction mixtures. Application of a purified conjugate-peptide reaction mixture to the iminobiotin column, resulted in the retention of the majority of ^{125}I activity. The small quantity of the label eluting on application of the reaction mixture could be attributed to complexes with no free biotin binding sites (i.e. tetramers). As the binding capacity of the column is specific for streptavidin then all retained ^{125}I

activity must be associated with the biotin-binding protein. Furthermore, the release of radiolabel from the column under conditions which increase the iminobiotin-streptavidin dissociation constant provides additional evidence that N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH was specifically retained through its link to streptavidin. In this regard, streptavidin effectively bridges between the biotinylated peptide ligand and iminobiotin residues on the column. This is possible as the protein consists of four homologous sub-units each containing a single biotin-binding site (Gitlin *et al*, 1988).

6.14.2. Purification of reaction mixtures.

The streptavidin-coated magnetic beads used in this study were found to be capable of specifically binding up to 442 picomoles of peptide per milligram of bead suspension. Therefore, using the method described in section 6.3.3. it was possible to remove up to 40% of the peptide included in the reaction mixture. However, it is clear from the data presented in Table 6.2. that over the 60 minute incubation period the majority of biotinylated peptide was conjugated to streptavidin and therefore remained in solution. The exact amount of free biotinylated peptide removed on the beads proved difficult to measure but importantly the quantity of ^{125}I activity retained from the reaction mixture was not significantly different to the level of non-specific binding. These data were indicative of a high efficiency of conjugation and suggested that a maximum of 1.5% of the peptide remained free. This was probably a reflection of the ratio at which streptavidin and the peptide were combined. Although equimolar quantities of streptavidin and biotinylated peptide were mixed in the reaction mixture the ratio of peptide-biotin residues to biotin-binding sites was 1 to 4. It is also noteworthy that these results show general agreement with the work of Chaiet and Wolf, (1964) who determined the biotin binding capacity of streptavidin using a microbiological assay. In this study when four molecules of biotin were combined with two molecules of streptavidin less than 1% of biotin remained free.

Encouraged by these results the ability of streptavidin-peptide conjugates to bind to the murine MC1 receptor was tested.

6.14.3. Binding of streptavidin-peptide conjugates to the MC1 receptor.

For targeting studies N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH was used as an alternative to α-MSH as it exhibits higher affinity binding at the MC1 receptor (Sawyer *et al*, 1980). The dissociation constant (K_{db}) for N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH (1.9 ± 1.3 nM) was similar to that estimated by Sahm *et al,* (1994) for [Nle^4,D-Phe^7]α-MSH (K_{db}= 2.02 nM). Therefore, after modification of the N-terminus of [Nle^4,D-Phe^7]α-MSH, to provide a biotin residue for linking to streptavidin, the peptide retains a high binding affinity. Conjugation of N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH to streptavidin and FITC-streptavidin produced species which retained the ability to displace [^{125}I-Tyr^2,Nle^4,D-Phe^7]α-MSH from the MC1 receptor; this radioligand binds specifically to the cell surface receptor (Sahm, 1994). However, the binding affinities of streptavidin-N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH (K_{db} = 75 ± 3.3 nM) and FITC-streptavidin-N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH (K_{db} = 81 ± 2.5 nM) were reduced compared to the native peptide. This decrease is thought to result from steric hindrance produced by conjugating the 2 kDa peptide to a large protein of approximately 60 kDa. However, the binding affinity of the complex was sufficiently high to proceed with targeting experiments. Indeed, biotinylated peptides have been used in the localisation of other G-protein coupled receptors (Schvartz *et al*, 1991; Sakai *et al*, 1994).

6.14.4. Gene transfer mediated by N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH.

Previous studies have shown transferrin containing molecular conjugate vectors to be capable of transfecting several cell types (Zenke *et al*, 1990; Lozier *et al*, 1994). B16 murine melanoma cells have been successfully transfected using this system (Wagner *et al*, 1994b). However, although transferrin receptors are overexpressed in

specific disease states (Faulk *et al*, 1980) these receptors are also found in high numbers on the cells of healthy tissues. Therefore, N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH was incorporated into complexes in an attempt deliver complexes through the MC1 receptor and increase the specificity of gene transfer. The expression of this type of G-protein coupled receptor is confined to melanocyte derived cells. However, in transfection experiments no increase in delivery efficiency was produced on incorporating N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH into complexes. This was surprising as it had previously been demonstrated that conjugation of these ligands to streptavidin did not ablate interaction with the receptor. However, under the conditions described in these experiments it is possible that although the receptor is internalised (Adams *et al*, 1993) the paucity of receptors on each cell limits the efficiency of gene delivery. The B16 cells used in this laboratory express between 5,000 to 25000 receptors/cell (Sahm, 1994). This is significantly lower than the density of transferrin receptors on the K-562 cell line used in studies with transferrin-polylysine conjugates; Wagner *et al*, (1994a) calculated that each cell K-562 cell presented 150,000 transferrin receptors. Furthermore, these workers have shown that the number of transferrin receptors significantly influences gene delivery efficiency (Cotten *et al*, 1990); up-regulation of transferrin receptors on K-562 cells with desferrioxamine increased gene transfer efficiency ten-fold. In addition, recent studies have shown uptake of transferrin-polycation-DNA complexes varied with the cell type. This was presumably a function of the receptor number (Plank *et al*, 1994; Curiel *et al*, 1994). In the case of the B16 melanoma cell line, incorporation of transferrin into polycation-DNA complexes produced only a 1.5-fold increase in luciferase gene expression.

6.14.5. Gene transfer mediated by concanavalin A.

Lectins are a group of proteinaceous molecules which are capable of recognising specific glycoconjugates on the surface of cells (Liener *et al*, 1986). The use of these

agents in targeting complexes to neoplastic cells is based on observations that cell surface differences exist between the normal and malignant phenotypes. The transformation from normal cells is associated with changes in the amount and/or display of cell surface glycoconjugates (Irimura and Nicolson, 1984). Additionally, on binding to their receptors lectins become internalised so that agents conjugated to these ligands are able to penetrate the plasma membrane of cells (Roche *et al*, 1983). Concanavalin A, which shows binding specificity to α-D-glucose and α-D-mannose expressing carbohydrate chains, was chosen as the targeting agent in this study as it has been successfully used in the receptor mediated delivery of chimeric toxins (Gilliland *et al*, 1978) and cationic-polypeptide DNA vectors to neoplastic cells (Batra *et al*, 1994). This molecule was shown to bind glycoconjugates on the cell surface of B16 cells with a high degree of specificity.

The inclusion of Con A in polycation-DNA complexes did produced a statistically significant increase in the delivery of polylysine-DNA complexes to B16 melanoma cells. However, this increase in β-galactosidase expression was only two fold greater than seen with the native complex and was associated with some variability between samples. The results obtained in this study were similar to those described by Batra *et al*, (1994) for delivery of polycation-DNA conjugates to Lewis lung carcinoma cells. Additionally, we must consider that although it was possible to increase the efficiency of delivery using this strategy a significant proportion of uptake was mediated through the electrostatic interaction of complexes with cell surfaces. Furthermore, following transfection with Con A targeted systems β-galactosidase expression in B16 cells was no greater than the level of enzyme activity routinely produced by polycation-mediated non-specific gene delivery (ca. 600 milliunits/mg protein). Therefore, although lectins can mediate gene delivery, the levels of efficiency achieved may not be practically significant.

6.15. Summary.

Targeting of polycation-DNA complexes would restrict the expression of therapeutic genes to neoplastic cells and reduce potential adverse effects from immuno- and gene directed enzyme prodrug therapy. In this study, two strategies were tested for the selective delivery of polycation-DNA complexes to melanoma cells; in each case biotinylated derivatives of the ligand were incorporated into streptavidin-polylysine conjugates. Complexes containing the peptide N^{α}-biotin-(Gly_3)-[Nle^4,D-Phe^7]α-MSH, found on melanocytes, failed to increase gene transfer efficiency. A less selective strategy which employed the lectin concanavalin A was more successful. However, the levels of β-galactosidase activity produced by this system were no greater than seen with the polycation-DNA complexes optimised for non-specific gene transfer. Therefore, the use of these targeted systems may be limited.

Chapter 7

Concluding discussion.

There are now more than 4500 human diseases which are classified as having a genetic origin (Mckusik, 1988). In the main, genetic disorders are relatively rare but it is now recognised that some of the most common illnesses such as cancers and cardiovascular disease have genetic components. These diseases are potential targets for gene therapies. However, in any treatment strategy the therapeutic gene must be introduced efficiently into the target tissue or cell. This makes the delivery system an active component of the therapy. On the basis of their potential for targeting and ability to encapsulate large sequences of foreign DNA, cationic polypeptides have emerged as promising non-viral technologies for gene transfer. However, if these systems are to form the basis of gene medicines, an understanding of the formulation factors controlling the efficiency of delivery and targeting is required.

The addition of cationic polypeptides to negatively charged DNA molecules is known to result in a rapid and spontaneous ionic interaction. Furthermore, the resultant charge neutralisation produces a thermodynamic state in which DNA molecules can condense into discrete structures. However, in order to produce pharmaceutically acceptable delivery systems it is necessary to better understand the factors affecting gene transfer. The most influential formulation factor appears to be the ratio of peptide cation-to-DNA phosphate in the system, which controls (1) the surface charge on complexes; (2) DNA condensation; and (3) complex morphology. Perhaps the most significant of these physicochemical properties is the electrostatic charge on complexes as it directly affects both non-specific and receptor-mediated gene transfer. In order to facilitate non-specific gene transfer the peptide cation/DNA phosphate ratio must be manipulated to produce complexes with a

positive zeta potential. Under these conditions positively charged complexes, like other cationic macromolecules (Ryser, 1967), are taken up into cells by endocytosis. However, the ideal system for *in vivo* targeted gene delivery would be electrostatically neutral; under these conditions DNA would be condensed, protected from nuclease digestion, and non-specific uptake minimised. Systems of this type have proved difficult to prepare by the method used in this study; the direct addition of polypeptides to DNA. Therefore, in targeting delivery of DNA through a cell surface receptor ligand a component of total gene transfer was charge mediated. Moreover, where the receptor ligand chosen for targeting is inefficient (e.g. concanavalin A), the level of gene transfer with ligand-polylysine complexes may be lower than that observed with a non-ligand-polypeptide system. In this respect, it may be more appropriate to deliver foreign genes using a non-specific delivery system whilst restricting expression with cell specific promoters.

It is possible that improvements in gene transfer efficiency can be achieved by modifying the physical and chemical nature of the polypeptide carrier. For example, in this study a critical dependence of the gene transfer efficiency on the molecular weight of the carrier was established. In polylysine based systems it is possible to manufacture a two-fold increase in gene transfer efficiency by the relatively simple measure of reducing the molecular weight of the carrier from 28,000 to 16,300 Da. A change in the conformation of the complex leading to reduced cross-linking may provide one possible explanation for the higher level of gene expression observed with low molecular weight carriers.

Recent studies have also proposed the inclusion into delivery systems of peptide sequences that encode nuclear localisation signals . The object of this strategy is to increase the amount of transgenic DNA reaching the nucleus (Conary *et al*, 1995). In this respect it is possible that the high levels of transformation achieved with histone H1 were the result of this protein carrying a nuclear localisation sequence.

The modification of the polypeptide sequence is also likely to be important for the acceptance of polypeptide carriers for *in vivo* gene transfer. Poly-L-lysine, although a useful model, is likely to be restricted by its inherent cytotoxicity. The cationic nature of this molecule causes it to adsorb to electronegative regions of membranes, a process which results in morphological changes and changes in cell permeability to ions and macromolecules (Ryser and Hancock, 1965). As an alternative, co-polymers containing neutral amino acid residues have been proposed as carriers. The substitution of lysine homopolymers with alanine produces branched co-polymers with reduced cytotoxicity and the ability to condense DNA. However, the results from transfection studies with these polymers were disappointing as genes delivered by this strategy were not expressed. More encouraging results have been observed with linear co-polymers of these amino acids (B. Thomas, personal communication). Since both linear and branched co-polymers of alanine and lysine induce DNA condensation, the differences in transfection activities are more likely to result from subtle changes in complex morphology or stability to nuclease enzymes.

In spite of the advantages offered by cationic polypeptide delivery systems the technique is still severely limited by the poor escape of endocytosed DNA from the endosome/lysosme trafficking pathway. Methods proposed to increase delivery efficiencies have included the co-internalisation of inactivated adenoviral particles and virally-derived fusion peptides. However, such techniques remain at the experimental stage and their safety *in vivo* has not been established. An alternative approach is the possible development of hybrid vector systems containing both a polycationic moiety and a membrane fusion-promoting lipid. Notably, a series of lipospermines has been synthesised and shown to be active *in vitro* (Behr *et al*, 1989). A more flexible strategy would be the inclusion of membrane active lipids into systems partially condensed with a non-toxic polycation. DNA condensed with the polycation is expected to be highly stabilised. In this respect Gershon *et al*,

(1993), in examining the conformation of the liposome-DNA complex have demonstrated the possibility of condensing DNA using simple mixtures of lipids and polycations. However, the gene transfer activity of such systems remains to be investigated.

Identification of some of the physicochemical factors affecting polycation mediated gene delivery represents a contribution to the rational design of non-viral gene delivery vectors. Moreover, it is envisaged that data obtained from model systems based on poly-L-lysine could be applied to increase the efficiency of less toxic second-generation cationic polypeptide carriers. In establishing the importance of physicochemical factors to the efficiency of gene transfer, their quantification and control is recognised as important to the development of cationic polypeptide-DNA complexes as gene medicines.

7.1. Further work.

At present, systematic efforts to show chemical structure activity relationships for cationic polypeptide carriers have been limited. Investigations of this type would constitute a major step towards the understanding of DNA complexation and the development of more efficient vector systems. On a more basic level, the methods used to prepare polypeptide-DNA complexes have been recognised as a potential cause of variations in gene transfer efficiency. The formation of complexes by a controlled annealing process, such as in salt gradient dialysis, may reduce problems of this kind. Further to the work presented in this study, more detailed knowledge of the correlation between the physicochemical properties of complexes and gene transfer would undoubtedly contribute to the development of more efficient vector systems.

References

Adams, G., Olivier, G.W.J., Branch, S.K., Moss, S.H., Notarianni, L.J., and Pouton, C.W. (1992) Evidence for the internalisation of [^{125}I-Tyr^{2},Nle^{4},D-Phe^{7}]α-MSH following binding to the MSH receptor of B16 murine melanoma cells, Ann.N.Y.Acad.Sci., **680**, 440-441.

Akrinrimisi., E.O., Bonner, J., and Ts'o, P.O.P. (1965) Binding of basic proteins to DNA, J.Mol.Biol., **11**, 128-136.

Ali, M., Lemoine, N.R., and Ring, C.J.A. (1994) The use of DNA viruses as vectors for gene therapy, Gene Therapy, **1**, 367-384.

Alton, E.F.W., Middleton, P.G., Caplen, N.J., Smith, S.N., Steel, D.M., Munkonge, F.M., Jeffry, P.K., *et al*, (1993) Non-invasive liposome-mediated gene delivery can correct the ion transport defect in cystic fibrosis mutant mice, Nat.Genetics, **5**, 135-142.

Anderson, W.F. (1992) Human gene therapy, Science, **256**, 808-809.

Anderson, W.F. (1993) What about those monkeys that got T-cell lymphoma ? (Editorial), Hum. Gene Ther., **4**, 1-2.

Andreason, G.L., and Evans, G.A. (1988) Introduction and expression of DNA molecules in eukaryotic cells by electroporation, Biotechniques, 6, 650-660.

Armentano, D., Sookdeo, C., and White, G., Giuggio, D., Souza, D., Couture, L., Cardoza, L., Vincent, K., Wadworth, S., and Smith, A. (1994) Second generation adenovirus vectors for cystic fibrosis gene therapy, J.Cell. Biochem., **18A (Suppl.)**, Abstract DZ102.

Bard, D.R., Knight, C.G., and Page-Thomas, D.P. (1990) A chelating derivative of α-melanocyte stimulating hormone as a potential imaging agent for malignant melanoma, Br.J.Cancer, **62**, 919-922.

Basu, S.K., Goldstein, J.L., Anderson, R.G.W., and Brown, M.S. (1981) Monensin interrupts recycling of low density lipoprotein receptors in human fibroblasts, Cell, **24**, 493-502.

Batra, R.K., Johanning, F.W., Wagner, E., Garver, R.I. and Curiel, D.T. (1994) Receptor mediated gene delivery employing lectin-binding specificity, Gene Therapy, **1**, 255-260.

Bauminger, S., and Wilchek, M., (1980) The use of carbodiimides in the preparation of immunizing conjugates, Methods Enzymol., **70**, 151-159.

Bayer, E.A., Ben-Hur, H., Gitlin, G., and Wilchek, M. (1986) An improved method for the single-step purification of streptavidin, J.Biochem.Biophys.Meth., **13**, 103-112.

Bayer, E.A., and Wilchek, M. (1990) Avidin- and strepatvidin containing probes, Methods Enzymol., **184**, 174-184.

Behr, J-P., Demeneix, B., Loeffler, J-P., and Perez-Mutul., J. (1989) Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA, Proc.Natl.Acad.Sci.USA, **86**, 6982-6986.

Bell, G.I. (1978) Models for the the specific adhesion of cells to cells, Science, **200**, 618-627.

Berkner, K.L. (1988) Development of adenovirus vectors for the expression of heterologous genes, Biotechniques, **6**, 616-629.

Birnboim, H.C., and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA, Nucl. Acids Res., **7**, 1513-1523.

Blackburn, S. (1968) Amino acid determination, Edward Arnold Ltd., London.

Bond V.C. and Wold B. (1987) Poly-L-ornithine-mediated transformation of mammalian cells, Mol.Cell.Biol., **7**, 2286-2293.

Bonner, W.M. (1975) Protein migration into nuclei. I. Frog oocyte nuclei *in vivo* accumulate microinjected histones, allow entry to small proteins, and exclude large proteins, J.Cell.Biol., **64**, 431-437.

Bradbury, E.M., Chapman, G.E., Danby, S.E., Hartman, P.G., and Riches, P.L. (1975a) Studies on the role and mode of operation of the very-lysine rich histone H1 (F1) in eukaryote chromatin; The properties of the N-terminal and C-terminal halves of histone H1, Eur.J.Biochem., **57**, 521-528.

Bradbury, E.M., Cary, P.D., Chapman, G.E., Crane-Robinson, C., Danby, S.E., Rattle, H.W.E., Boublik, M., Palau, J. and Aviles, F.J. (1975b) Studies on the role and mode of operation of the very-lysine rich histone H1(F1) in eukaryote chromatin; The conformation of histone H1, Eur.J.Biochem., **52**, 605-613.

Breeuwer, M., and Goldfarb, D.S. (1990) Facilitated nuclear transport of Histone H1 and other small nucleophilic proteins, Cell, **60**, 999-1008.

Bullock, W.O., Fernandez, J.M., and Short, J.M. (1987) XL1-Blue: A high efficiency plasmid transforming recA Eschericia coli strain with β-galactosidase selection, Biotechniques, **5**, 376-379.

Caplen., N.J., Alton, E.W.F.W., Middleton, P.G., Dorin, J.R., Stevenson, B.J., Gao, X., Durham, S.R. *et al*, (1995) Lipososme-mediated CFTR gene transfer to the nasal epithelia of patients with cystic fibrosis, Nat.Medicine, **1**, 39-46.

Carrol, D. (1972) Complexes of polylysine with polyuridylic acid and other polynucleotides, Biochemistry, **11**, 426-433.

Chaiet, L., and Wolf, F.J. (1964) The properties of streptavidin, a biotin-binding protein produced by streptomycetes. Arch.Biochem.Biophys., **106**, 1-5.

Chang, C., Weiskopf, M., and Li, H.J. (1973) Conformational studies of nucleoprotein. Circular dichroism of deoxyribonucleic acid base pairs bound by polylysine, Biochemistry, **12**, 3028-3031.

Chen, J., Gamou, S., Takayanagi, A., and Shizmizu, N. (1994) A novel gene delivery system using EGF receptor mediated endocytosis, FEBS letters, **338**, 167-169.

Chen, J., Stickles, R.J. and Daichendt, K.A. (1994) Galactosylated histone-mediated gene transfer and expression, Hum. Gene Ther., **5**, 429-435.

Chiou, H.C., Tangco, M.V., Levine, S.M., Robertson, D., Kormis, K., Wu, C.H., and Wu, G.Y. (1994) Enhanced resistance to nuclease degradation of nucleic acids complexed to asialoglycoprotein-polylysine carriers, Nucl.Acids Res., **22**, 5439-5446.

Chowdury, N.M., Wu, C.H., Wu, G.Y., Yerneni, P.C., Bommineni, V.R., and Chowdury, J.R. (1993) Fate of DNA targeted to the liver by asialoglycoprotein receptor-mediated endocytosis *in vivo*, J.Biol.Chem., **268**, 11265-11271.

Clark, D.J., and Thomas, J.O. (1986) Salt-dependent co-operative interaction of histone H1 with linear DNA, J.Mol.Biol., **187**, 569-580.

Cohen, S.N., Chang, A.C.Y., and Hsu, L. (1972) Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of *Escherichia coli* by R-factor DNA, Proc.Natl.Acad.Sci.USA, **69**, 2110-2114.

Conary, J.T., Faulks, R.D., Gagne, L., Price, P., Kon, V., Canonico, A.E., Christman, B.W., Erdos, G., Brigham, K.L., and Schreier, H. (1995) Use of synthetic peptides to enhance transgene expression, Proceed.Intern.Symp.Control.Rel.Bioact.Mater., **22**, 436-437.

Cotten, M., Längle-Rouault, F., Kirlappos, H., Wagner, E., Mechtler, K., Zenke, M., Beug, H., and Birnstiel, M.L. (1990) Transferrin-polycation-mediated introduction of DNA into human leukemic cells: Stimulation by agents that affect the survival of transfected DNA or modulate transferrin receptor levels, Proc.Natl.Acad.Sci.USA, **87**, 4033-4037.

Cotten, M., Wagner, E., Zatloukal, K., Phillips, S., Curiel, D.T., and Birnstiel, M.L. (1992) High-efficiency receptor-mediated delivery of small and large (48 kilobase gene constructs) using the endosome-disruption activity of defective or chemically inactivated adenovirus particles, Proc.Natl.Acad.Sci.USA, **89**, 6094-6098.

Cotten, M., Wagner, E., and Birnstiel, M.L. (1993) Receptor-mediated transport of DNA into eukaryotic cells, Methods.Enzymol., **217**, 618-644.

Cotten, M., Baker, A., Saltik, M., Wagner, E., and Buschle, M. (1994) Lipopolysacchride is a frequent contaminant of plasmid DNA preparations and can be toxic to primary human cells in the presence of adenovirus, Gene Therapy, **1**, 239-246.

Cox, R.A., and Gokcen, M. (1976) Comparison of serum DNA, native DNA-binding and deoxyribonuclease levels in ten animal species and man, Life Sciences, **19**, 1609-1614.

Cox, G.A., Cole, N.M., Matsumura, K., Phelps, S.F., Hauschka, S.D., Campbell, K.P., Faulkner, J.A., and Chamberlain, J.S. (1993) Overexpression of dystrophin in transgenic mdx mice eliminates dystrophic symptoms without toxicity, Nature, **364**, 725-729.

Cristiano, R.J., Smith, L.C., and Woo, S.L.C. (1993) Hepatic gene therapy: Adenovirus enhancement of receptor-mediated gene delivery and expression in primary hepatocytes, Proc.Natl.Acad.Sci.USA, **90**, 2122-2126.

Crystal, R.G., Mcelvaney, N.G., Rosenfeld, M.A., Chu, C.S., Mastrangeli, A., Hay, J.G., Brody, S.L., Jaffe, H.A., and Danel, C. (1994) Administration of an adenovirus containing the human CFTR cDNA to the respiratory tract of individuals with cystic fibrosis, Nature Genet., **5**, 397-402.

Curiel, D.T., Agarwal., S., Wagner, E., and Cotten, M. (1991) Adenovirus enhancement of transferrin-polylysine-mediated gene delivery, Proc.Natl.Acad.Sci.USA, **88**, 8850-8854.

Curiel, D.T. (1994) High efficiency gene transfer employing adenovirus-polylysine-DNA complexes, Nat.Immun., **13**, 141-164.

Curiel, T.Y., Cook, D.R., Bogedain, C., Jilg, W., Harrison, G.S., Cotten, M., Curiel, D.T., and Wagner, E. (1994) Efficient foreign gene expression in Epstein-Barr virus-transformed human B-cells, Virology, **198**, 577-585.

Dalgleish, A.G. (1994) Cancer vaccines, Eur.J.Cancer, **30A**, 1029-1035.

Danielsen, M., Northrop, J.P., and Ringold, G.M. (1986) The mouse glucocorticoid receptor: mapping functional domains by cloning, sequencing, and expression of wild-type mutant receptor proteins, EMBO.J., **5**, 2513-2522.

Davis, H.L., Whalen, R.G., and Demeneix, B.A. (1993) Direct gene transfer into skeletal muscle *in vivo*: factors affecting efficiency of transfer and stability of expression, Hum.Gene Ther., **4**, 151-159.

Davies, K. (1992) Moving straight to the target, Nature, **358**, 519.

Dean, R.T., Jessup, W., and Roberts, C.R. (1984) Effects of exogenous amines on mammalian cells, with particular reference to membrane flow, Biochem.J., **217**, 27-40.

Deonarain, M.P., Spooner, R.A., and Epenetos, A.A. (1995) Genetic delivery of enzymes for cancer therapy, Gene Therapy, **2**, 235-244.

Dillon, N. (1993) Regulating gene expression in gene therapy, Trends in Biotechnology, **11**, 167-172.

Dingwall, C., and Allan, J. (1984) Accumulation of the isolated carboxy-terminal domain of histone H1 in the Xenopus Oocyte nucleus, EMBO.J., **3**, 1933-1937.

Donahue, R.E., Kessler, S.W., Bodine, D., McDonagh, K., Dunbar, C., Goodman, S., Agricola, B., Byrne, E., Raffeld, M., Moen, R., Bacher, J., Zsebo, K.M., and Neinhuis, A.W. (1992) Helper virus induced T-cell lymphoma in nonhuman primates after retroviral mediated gene transfer, J.Exp.Med., **176**, 1125-1135.

Duzgunes, N., Goldstein, J.A., Friend, D.S., and Felgner, P.L. (1989) Fusion of liposomes containing a novel cationic lipid N-[2,3-(dioleyloxy)propyl]-N,N,N,-trimethylammonium: Induction by multivalent anions and asymmetric fusion with acidic phospholipid vesicles, Biochemistry, **28**, 9179-9184.

Eberle, A.N. (1988) The melanotropins: chemistry, physiology and mechanisms of action. Karger Press, Basel.

Eberle, A.N., and Hübscher, W. (1979) α-Melanotropin labelled at its tyrosine^2 residue: Synthesis of 3'iodotyrosine^2, 3-^{125}-iodotyrosine^2 and (3',5'-$^3H_2$)tyrosine^2-α-melanotropin and of related peptides, Helv.Chim.Acta, **62**, 2469-2483.

Eglitis, M.A., and French Anderson, W. (1988) Retroviral vectors for introduction of genes into mammalian cells, Biotechniques, **6**, 608-614.

Ennever, J.F., Love, S.M., and Harpst, J.A. (1985) Ionic effects on the structure of nucleoprotein cores from adenovirus, Biochim.Biophys.Acta, **826**, 67-79.

Erskine-Grout, M.B. (1993) Interaction of alpha-melanocyte-stimulating hormone with its receptor, PhD thesis, University of Bath.

Etienne-Julan, M., Roux, P., Carillo, S., Jeanteur, P., and Piechaczyk, M. (1992) The efficiency of cell targeting by recombinant retroviruses depends on the nature of the receptor and the composition of the artificial cell-virus linker, J.Gen.Virol., **73**, 3251-3255.

Farber, F.E., Melnick, J.L., and Butel, J.S. (1975) Optimal conditions for uptake of exogenous DNA by Chinese hamster lung cells deficient in hypoxanthine-guanine phosphoribosyltransferase, Biochim.Biophys.Acta, **390**, 298-311.

Faulk, W.P., Li-Hsi, B., and Stevens, P. (1980) Transferrin and transferrin receptors in carcinoma of the breast, Lancet, **2**, 390-392.

Fearon, E.R., Pardoll, D.M., Itaya, T., Golumbek, P., Levitsky, H.I., Simons, J.W., Karasuyama, H., Vogelstein, B., and Frost, P. (1990) Interleukin-2 production by tumor cells bypasses T-helper cell function in the generation of antitumor response, Cell, **60**, 1397-1403.

Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringolad, G.M., and Danielsen, M. (1987) Lipofection: A highly efficient, lipid mediated DNA-transfection procedure, Proc.Natl.Acad.Sci.USA, **84**, 7413-7417.

Felgner, P.L. (1990) Particulate systems and polymers for *in vitro* and *in vivo* delivery of polynucleotides, Adv.Drug Del.Rev., **5**, 163-187.

Felgner, P.L., and Rhodes, G. (1991) Gene therapeutics, Nature, **349**, 351-352.

Felgner, J.H., Kumar, R., Sridhar, C.N., Wheeler, C.J., Tsai, Y.J., Border, R., Ramsey, P., Martin, M., and Felgner, P.L. (1994) Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations, J.Biol.Chem., **269**, 2550-2564.

Findeis, M.A., Merwin, J.R., Spitalny, G.L., and Chiou, H.C. (1993) Targeted delivery of DNA for gene therapy via receptors, Trends in Biotechnology, **11**, 202-205.

Flotte, T.R. and Carter, B.J. (1995) Adeno-associated virus vectors for gene therapy, Gene Therapy, **2**, 357-362.

Foa, R., Guarini, A., and Gansbacher, B. (1992) IL2 treatment for cancer: from biology to gene therapy, Br.J.Cancer, **66**, 992-998.

Freese, A., and Geller, A.L., (1993) Infection of cultured striatal neurons with a defective HSV-1 vector: implications for gene therapy, Nucleic Acids Res., **19**, 7219-7223.

Friedmann, T. (1993) Gene therapy-a new kind of medicine, Trends in Biotechnology, **11**, 156-158.

Fudem-Goldin, B., and Orr, G.A. (1990) 2-iminobiotin containing reagent and affinity columns, Methods Enzymol., **184**, 167-173.

Gansbacher, B., Zier, K., Daniels, B., Cronin, K., Bannerji, R., and Gilboa, E. (1990) Interleukin 2 gene transfer into tumor cells abrogates tumorigenicity and induces protective immunity, J.Exp.Med., **172**, 1217-1224.

Gao, L., Wagner, E., Cotten, M., Agarwal, S., Harris, C., Romer, M., Miller, L., Hu, P.-C., and Curiel, D.T. (1993) Direct *in vivo* gene transfer to airway epithelium employing adenovirus-polylysine-DNA complexes, Hum. Gene Ther., **4**, 17-24.

Gao, X., and Huang, L. (1991) A novel cationic liposome reagent for efficient transfection of mammalian cells, Biochim.Biophys.Acta., **179**, 280-285.

Geller, A.L., and Breakefield, X.O. (1990) A defective HSV-1 vector expresses Escherichia coli β-galactosidase in cultured peripheral neurons, Science, **241**, 1667-1669.

Gershon, H., Ghirlando, R., Guttman, S.B., and Minsky, A. (1993) Mode of formation and structural features of DNA-cationic liposome complexes used for transfection, Biochemistry, **32**, 7143-7151.

Gilliland, D.G., Collier, R.J., Moehring, J.M., and Moehring, T.J. (1978) Chimeric toxins: toxic, disulfide-linked conjugate of concanavalin A with fragment A from diptheria toxin, Proc.Natl.Acad.Sci.USA, **75**, 5319-5323.

Gitlin, G., Bayer, E.A., and Wilchek, M. (1988) Studies on the biotin-binding site of streptavidin, Biochem.J., **256**, 279-292.

Gottschalk, S., Cristiano, R.J., Smith, L.C., and Woo, S.L.C. (1994) Folate receptor mediated DNA delivery into tumour cells: potosomal disruption results in enhanced gene expression, Gene Therapy, **1**, 185-191.

Graessmann, M., and Graessmann, A. (1986) Microinjection and organelle transplantation, Academia, London, UK.

Graham, F.L., and Van der Eb, A.J. (1978) A new technique for the assay of infectivity of human adenovirus 5, Virology, **52**, 456-467.

Greaves, D.R., Wilson, F.D., Lang, G., and Kioussis, D. (1989) Human CD2 3'-flanking sequence confer high level, T cell-specific, position independent gene expression in transgeneic mice, Cell, **56**, 979-986.

Green, N.M. (1966) Thermodynamics of the binding of biotin and some analogues by avidin, Biochem, J., **101**, 774-780.

Gutierrez, A.A., Lemoine, N.R., and Sikora, K. (1992) Gene therapy for cancer, Lancet, **339**, 715-721.

Haensler, J., and Szoka, F.C. (1993) Polyamido acid cascade polymers mediate efficient transfection of cells in culture, Bioconjugate Chem., **4**, 372-379.

Hanover, G.T., and Dickson, R.B. (1985) Endocytosis, Plaenum Press, New York.

Harris, J.D., Gutierrez, A.A., Hurst, H.C., Sikora, K., and Lemoine, N.R. (1994) Gene therapy for cancer using tumour-specific prodrug activation, Gene Therapy, **1**, 170-175.

Harris, J.L., and Lerner, A.B. (1957) Amino acid sequence of the α-melanocyte stimulating hormone, Nature, **179**, 1346-1347.

Hodgson, C.P. (1995) The vector void in gene therapy, Biotechnology, **13**, 222-225.

Hofmann, K., Wood, S.W., Brinton, C.C., Montibeller, J.A., and Finn, F.M. (1980) Iminobiotin affinity columns and their application to retrieval of streptavidin, Proc.Natl.Acad.Sci.USA, **77**, 4666-4668.

Horn, N.A., Meek, J.A., Budahazi, G., and Marquet, M. (1995) Cancer gene therapy using plasmid DNA: Purification of DNA for human clinical trials, Hum. Gene Ther., **6**, 565-573.

Huber, B.E., Richards, C.A., and Krenitsky, T.A. (1991) Retroviral-mediated gene therapy for the treatment of hepatocellular carcinoma: An innovative approach for cancer therapy, Proc.Natl.Acad.Sci.USA, **88**, 8039-8043.

Huebers, H.A., and Finch, C.A. (1987) The physiology of transferrin and transferrin receptors, Physiol.Rev., **67**, 520-581.

Hunter, R.J. (1981) Zeta potential in colloid science, Academic Press, London, UK.

Hyde, S.C., Gill, D.R., Higgins, C.F., Trezise, A.E.O., MacVinish, L.J., Cuthbert, A.W., Ratcliff, R., Evans, M.J., and Colledge, W.H. (1993) Correction of the ion transport defect in cystic fibrosis transgenic mice by gene therapy, Nature, **362**, 250-255.

Hynes, R.O. (1976) Cell surface proteins and malignant transformation, Biochim.Biophys. Acta, **458**, 73-107.

Irimura, T., and Nicolson, G.L. (1984) Carbohydrate chain analysis by lectin binding to electrophoretically separated glycoproteins from murine B16 melanoma sublines of various metastatic properties, Cancer Res., **44**, 791-798.

Jaffe, H.A., Danel, C., Longnecker, G., Metzger, M., Setoguchi, Y., Rosenfeld, M.A., Gant, T.W. *et al,* (1992) Adenovirus-mediated *in vivo* gene transfer and expression in normal rat liver, Nat.Genet., **1**, 372-378.

Johnson, C.A., Goddard, J.P., and Adams, R.L.P. (1995) The effect of histone H1 and DNA methylation on transcription, Biochem.J., **305**, 791-798.

Johnston, S.A. (1990) Biolistic transformation: microbes to mice, Nature, **346**, 776-777.

Kaneda,Y., Iwai, K., and Uchida, T. (1989) Increased expression of DNA co-introduced with nuclear protein in adult rat liver, Science, **243**, 375-378.

Kass-Eisler, A., Falck-Pedersen, E., Elfenbein, D.H., Alvira, M., Buttrick, P.M., and Leinwand, L.A. (1994) The impact of development stage, route of administration and the immune system on adenovirus mediated gene transfer, Gene Therapy, **1**, 395-402.

Kawabata, K., Takakura, Y., and Hashida, M. (1995) The fate of plasmid DNA after intravenous injection in mice: Involvement of scavenger receptors in hepatic uptake, Pharm.Res., **12**, 825-830.

Kawai., S., and Nishizawa, M. (1984) New procedure for DNA transfection with polycation and dimethylsulphoxide, Mol.Cell.Biol., **4**, 1172-1174.

Kay, M.A., Baley, P., Rothenberg, S., Leland, F., Fleming, L., Parker-Ponder, K., Liu, T-J., Finegold, M., Darlington, G., Pokorny, W., and Woo, S.L.C. (1992) Expression of human α_1-antitrypsin in dogs after autologous transplantation of retroviral transduced hepatocytes, Proc.Natl.Acad.Sci.USA, **89**, 89-93.

Koch, K.S., Fletcher, R.G., Grond, M.P., Inyang, A.I., Lu, X.P., Brenner, and Leffert, H.L. (1993) Inactivation of plasmid reporter gene expression by one benzo(a)pyrenediol-epoxide DNA adduct in adult rat hepatocytes, Cancer Res., **53**, 2279-2286.

Laemmli, U.K. (1975) Characterisation of DNA condensates induced by poly(ethyleneoxide) and polylysine, Proc.Natl.Acad.Sci.USA, **72**, 4288-4292.

Le Gal La Salle, G., Robert, J.J., and Bernard, S., (1993) An adenovirus vector for gene transfer into neurones and glia in the brain, Science, **259**, 998-990.

Ledley, T.M., and Ledley, F.D. (1994) Multicompartment, numerical model of cellular events in the pharmacokinetics of gene therapies, Hum. Gene Ther., **5**, 679-691.

Legendre, J.Y., and Szoka, F.C. (1992) Delivery of plasmid DNA into mammalian cell lines using pH-sensitive liposomes: Comparison with cationic liposomes, Pharm.Res., **9**, 1235-1242.

Lehmann, A.R., and Oomen, A. (1985) Effect of DNA damage on the expression of CAT after transfection, Nucl. Acids Res., **13**, 2087-2095.

Lejeune, F.J., and Ghanem, G.E. (1993) Attempts to use α-melanotropin-containing melphalan in melanoma patients, Ann. N.Y. Acad.Sci., **680**, 491-500.

Leng, M., and Felsenfeld, G. (1966) The preferential interactions of polylysine and polyarginine with specific base sequences in DNA, Proc.Natl.Acad.Sci.USA, **56**, 1325-1332.

Liener, I.E., Sharon, N., and Goldstein, I.J. (1986) The lectins. Properties, functions, and applications in biology and medicine, Academic Press, Orlando, USA.

Logan, J.L., Bebok, Z., Walker, L.C., Peng, S., Felgner, P.L., Siegal, G.P., Frizzell, R.A., Dong, J., Howard, M., Matalon, S., Lindsey, J.R., DuVall, M., and Sorscher, E.J. (1995) Cationic lipids for reporter gene and CFTR transfer to rat pulmonary epithelium, Gene Therapy, **2**, 38-49.

Lowry, O.H., Rosebrough, N.T., Lewis-Farr, A., and Randal, R.J. (1951) Protein measurement with the folin phenol reagent, J.Biol.Chem., **193**, 265-275.

Lozier, J.N., Thompson, A.R., Hu, P., Read, M., Brinkhous, K.M., High, K.A. and Curiel, D.T. (1994) Efficient transfection of primary cells in a canine hemophilia B model using adenovirus-polylysine-DNA complexes, Hum. Gene Ther., **5**, 313-322.

Luthmann, H., and Magnusson, G. (1983) High efficiency polyoma DNA transfection of chloroquine treated cells, Nucl.Acids Res., **11**, 1295-1308.

MacGregor, G.R., Nolan, G.P., Fiering, S., Roederer, M., and Herzenberg, L.A. (1991) Use of E.coli *lacZ* (β-galactosidase) as a reporter gene, Methods Mol.Biol., **7**, 217-235.

Madaio, M.P., Hodder, S., Schwartz, B, and Stoller, B.D. (1984) Responsiveness of autoimmune and normal mice to nucleic acid antigens. J.Immunol., 132, 872-876.

Malone, R.W., Felgner, P.L., and Verma, I.M. (1989) Cationic liposome-mediated RNA transfection, Proc.Natl.Acad.Sci.USA, **86**, 6077-6081.

Manning, G.S. (1981) The possibility of intrinsic local curvature in DNA toroids, Biopolymers, **20**, 1261-1270.

March, K.L., Madison, J.E., and Trapnell, B.C. (1995) Pharmacokinetics of adenoviral vector-mediated gene delivery to vascular smooth muscle cells: Modulation by poloxamer 407 and implications for cardiovascular gene therapy, Hum. Gene Ther., **6**, 41-53.

Martin, A.N., Swarbrick, J., and Camarata, A. (1970) Physical Pharmacy, 2nd edition, Lea and Febiger, Philadelphia, USA.

McGuire, J., McGill, R., Leeman, S., and Goodfriend, T. (1965) The experimental generation of antibodies to α-melanocyte stimulating hormone and adrenocoticotropic hormone, J.Clin. Invest., **44**, 1672-1678.

Mckusik, V.A. (1988) Mendelian inheritance in man, John Hopkins University Press, Baltimore.

Mercer, W.E., Shields, M.T., Lin, D., Appella, E., and Ulrich, S. (1991) Growth supression induced by wild type p53 protein is accompanied by selective down-regulation of proliferating cell nuclear antigen expression, Proc.Nat.Acad.Sci.USA, **88**, 1958-1962.

Michael, S.I., and Curiel, D.T. (1994) Strategies to achieve targeted gene delivery via the receptor-mediated endocytosis pathway, Gene Therapy, **1**, 223-232.

Midoux, P., Mendes, C., Legrand, A., Raimond, J., Mayer, R., Monsigny, M., and Roche, A.C. (1993) Specific gene transfer mediated by lactosylated poly-L-lysine into hepatoma cells, Nucl. Acids Res., **21**, 871-878.

Miller, J.H. (1972) Assay of β-galactosidase, Experiments in molecular genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Miller, D.G., Adam, M.A., and Miller, A.D. (1990) Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection, Mol.Cell.Biol., **10**, 4329-4242.

Mitani, K., and Caskey, C.T. (1993) Delivering therapeutic genes-matching approach and application, Trends in Biotechnology, **11**, 162-166.

Monsigny, M., Roche, A., Midoux, P., and Mayer, R. (1994) Glycoconjugtaes as carriers for specific delivery of therapeutic drugs and genes, Adv.Drug Del.Rev., **14**, 1-24.

Morandini, R., Süli-Varga, H., Liebert, A., Loir, B., Butyanski, J., Medzihradsky, K., and Ghanem, G. (1994) Receptor-mediated cytotoxicity of α-MSH fragments containing melphalan in a human melanoma cell line, Int.J.Cancer, **56**, 129-133.

Mostor, K.E., Friedlander, M., and Blobel, G. (1984) The receptor for transepithelial transport of IgA and IgM contains multiple immunoglobulin like domains, Nature, **308**, 37-43.

Mulligan, R.C., and Berg, P. (1981) Selection of animal cells that express the Eschericia coli gene coding for xanthine-guanine phosphoribosyl transferase, Proc.Natl.Acad.Sci.USA, **78**, 2072-2076.

Mumper, R.J., Wang, J., Claspell, J.M., and Rolland, A.P. (1995) Novel polymeric condensing carriers for gene delivery, Proceed.Intern.Symp.Control.Rel.Bioact.Mater., **22**, 178-179.

Nabel, E.G., Plautz, G., and Nabel, G.J. (1992) Transduction of a foreign histocompatibility gene into the arterial wall induces vasculitis, Proc.Natl.Acad.Sci.USA, **89**, 5157-5161.

Nicolson, G.L. (1982) Cancer metastasis: organ colonisation and the cell surface properties of malignant cells, Biochim.Biophys.Acta, **695**, 113-176.

Nolan, G.P., Fiering, S., Nicolas, J.-F., and Herzenberg, L.A. (1988) Fluorescence-activated cell analysis and sorting of viable mammalian cells based on β-D-galactosidase after transduction of E.coli *lacZ*, Proc.Natl.Acad.Sci.USA, **85**, 2603-2607.

Ogata, N., Ueda, K., Kagamiyama, H., and Hayaishi, O. (1980) ADP-ribosylation of histone H1, J.Biol.Chem., **255**, 7616-7617.

Ohashi, T., Boggs, S., Robbins, P., Bahnson, A., Patrene, K., Wei, F-S., Wei, J-F., Li, J., Lucht, L., Fei, Y., Clark, S., Kimak, M., He, H., Mowery-Rushton, P., and Barranger, J.A. (1992) Efficient transfer and sustained high expression of the human glucocerebrosidase gene in mice and their functional macrophages following transplantation of bone marrow transduced by a retroviral vector, Proc.Natl.Acad.Sci.USA, **89**, 11332-11336.

Olins, D.E., Olins, A.L., and Von Hippel, P.H. (1967) Model nucleoprotein complexes: Studies on the interaction of cationic homopolypeptides with DNA, J.Mol.Biol., **24**, 157-176.

Olins, D.E., Olins, A.L., and Hippel., P.H. (1968) On the structure and stability of DNA-protamine and DNA-polypeptide complexes, J.Mol.Biol., **33**, 265-281.
Olins, D.E. (1969) Interaction of lysine-rich histones and DNA, J.Mol.Biol., **43**, 439-460.

Olins, D.E. and Olins, A.L. (1971) Model nucleohistones: The interaction of F1 and F2a1 histones with native T7 DNA. J.Mol.Biol., **57**, 437-455.

Perales, J.C., Ferkol, T., Beegan, H., Ratnoff, O.D., and Hanson, R.W. (1994) Gene transfer *in vivo*: Sustained expression and regulation of genes introduced into the liver by receptor-targeted uptake, Proc.Natl.Acad.Sci.USA, **91**, 4086-4090.

Peters, R. (1986) Fluorescence microphotoysis to measure nucleocytoplasmic transport and intracellular mobility, Biochim.Biophys.Acta, 864, 305-359.

Plank, C., Oberhauser, B., Mechtler, K., Koch, C., and Wagner, E. (1994) The influence of endosome-disruptive peptides on gene transfer using synthetic virus-like gene transfer systems, J.Biol.Chem., **269**, 12918-12924.

Plautz, G.E., Yang, Z-Y, Wu, B-Y, Gao, X., Huang, L., and Nabel, G.J. (1993) Immunotherapy of malignancy by *in vivo* gene transfer into tumors, Proc.Natl.Acad.Sci.USA, **90**, 4645-4649.

Ragot, T., Vincent, N., Chafey, P., Vigne, E., Gilgenkrantz, H., Couton, D., Cartard, J., Briand, P., Kapell, J-C., Perricaudet, M., and Kahn, A. (1993) Efficient adenovirus mediated transfer of a human minidystrophin gene to skeletal muscle of mdx mice, Nature, **361**, 647-650.

Riordan, J.R., Rommens, J.M., Kerem, B-S., Alon, N., Rozmahel, R., Grzelchak, Z., Zielenski, J., *et al.* (1989) Identification of the cystic fibrosis gene: cloning and characterisation of complementary DNA, Science, **245**, 1066-1073.

Roche, A.C., Barzilay, M., Midoux, P., Junqua, S., Sharon, N., and Monsigny, M. (1983) Sugar-specific endocytosis of glycoproteins by Lewis lung carcinoma cells, J.Cell Biochem., **22**, 131-140.

Roche, A.C., Midoux, P., Pimpaneau, V., Negre, E., Mayer, R., and Monsigny, M. (1990) Endocytosis mediated by monocyte and macrophage membrane lectins-application to antiviral drug targeting, Res.Virol., **141**, 243-249.

Rojanasakul, Y., Wang, L.Y., Malanga, C.J., Ma, J.K.H., and Liaw, J. (1994) Targeted gene delivery to alveolar macrophages via Fc receptor mediated endocytosis, Pharm.Res., **11**, 1731-1736.

Rolland, A., Duguid, J., Barron, M., Gong, L., Levin, J., and Eastman, E. (1994) Characterisation of non-viral gene medicines, Proceed.Intern.Symp.Control.Rel.Bioact. Mater., **21**, 240-241.

Rosenberg, S.A., Aebersold, P., Cornetta, K., Kasid, A., Morgan, R.A., Moen, R., Karson, E.M., Lotze, M.T., *et al.* (1990) Gene transfer into humans-immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction, N.Eng.J.Med., 9, 570-578.

Rosenfeld, M.A., Yoshimura, K., Trapnell, B.C., Yoneyama, K., Rosenthal, E.R., Daleamns, W., Fukayama, M., *et al.* (1988) *In vivo* gene transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium, Cell, **68**, 143-155.

Rosenkranz, A.A., Yachmenev, S.V., Jans, D.A., Serebryakova, N.V., Muravev, V.I., Peters, R., and Sobolev, A.S. (1992) Receptor-mediated endocytosis and nuclear transport of a transfecting DNA construct, Exp.Cell.Res., **199**, 323-329.

Ryser, H.J.-P., and Hancock, R. (1965) Histones and basic polyamino acids stimulate the uptake of albumin by tumor cells in culture, Science, **150**, 501-503.

Ryser, H.J.P. (1967) A membrane effect of basic polymers dependent on molecular size, Nature, **215**, 934-936.

Ryser, H. J.-P, and Shen, W-C. (1978) Conjugation of methotrexate to poly(L-lysine) increases drug transport and overcomes drug resistance in cultured cells, Proc.Natl.Acad.Sci.USA, **75**, 3867-3870.

Sahm, U.G. (1994) Interaction of naturally occuring and synthetic MSH peptides with peripheral and CNS melanocortin receptors, PhD thesis, University of Bath.

Sahm, U.G., Olivier, G.W.J., Branch, S.K., Moss, S.H., and Pouton, C.W. (1994) Synthesis and biological evaluation of α-MSH analogues substituted with alanine, Peptides, **15**, 1297-1302.

Sakai. T., Satoh, M., Hayashi, H., Fujikura, K., Sano, I., Koyama, H., Tatemoto, K., and Itoh, Z. (1994) Biotinyl C-terminal extended motilin as a biologically active receptor probe, Peptides, **15**, 257-262.

Sambrook, J., Fritsch, E.F., and Manniatis, T. (1989) Molecular cloning. A laboratory manual. 2nd edition, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

San, H., Yang, Z-Y., Pompili, V.J., Jaffe, M.L., Plautz, G.E., Xu, L., Felgner, J.H., Wheeler, C.J., Felgner, P.L., Gao, X., Huang, L., Gordon, D., Nabel, G.J., and Nabel, E.G. (1993) Safety and short term toxicity of a novel cationic lipid formulation for human gene therapy, Hum. Gene Ther., **4**, 781-788.

Sawyer, T.K., Sanfilippo, P.J., Hruby, V.J., Engel, M.H., Heward, C.B., Burnett, J.B., and Hadley, M.E. (1980) 4-Norleucine, 7-D-phenylalanine-α-melanocyte stimulating hormone: A highly potent α-melanotropin with ultralong biological activity, Proc.Natl.Acad.Sci.USA, **77**, 5754-5758.

Schreier, H., Conary, J.T., Bock, T., Erdos, G.W., Canonico, A.E., Brigham, K.L., and Muller, B.W. (1994) Liposome mediated gene delivery: I. Physicochemical properties of cationic liposome DNA plasmid complexes, Proceed.Intern.Symp.Control.Rel.Bioact.Mater., **21**, 140-141.

Schreier, H. (1994) The new frontier: gene and oligonucleotide therapy, Pharm.Acta.Helv., **68**, 145-159.

Schrewe, H., Thompson, J., and Bona, M. (1990) Cloning of the complete gene for carcinoembryonic antigen: analysis of its promoter indicates a region conveying cell-type specific expression, Mol Cell Biol., **10**, 2738-2748.

Schvartz, I., Gitlin, G., Amarant, T., Ittoop, O., and Hazum, E. (1991) Biotinylated endothelin as a probe for the endothelin receptor, Peptides, **12**, 1229-1233.

Shapiro, J.T., Leng, M., and Felsenfeld, G. (1969) Deoxyribonucleic acid-polylysine complexes: Structure and nucleotide specificity, Biochemistry, **8**, 3219-3232.

Sheehan, J.C., and Hess, G.P. (1955) A new method of forming peptide bonds, J.Am.Chem.Soc., **77**, 1067-1068.

Sheldon, K., Baumal, R., and Marks, A. (1992) Targeting of [^{111}In]Biocytin to cultured ovarian adenocarcinoma cells using covalent monoclonal antibody streptavidin conjugates, Int.J.Radiat.Appl.Instrum., **43**, 1399-1402.

Shimamoto, C., Weinstein, W.M., and Boland, C.R. (1987) Glycoconjugate expression in normal, metaplastic, and neoplastic human upper gastrointestinal mucosa, J.Clin.Invest., **80**, 1670-1687.

Shimotohmo, K., and Temin, H.M. (1981) Formation of infectious progeny virus after insertion of Herpes simplex thymidine kinase gene into DNA of avian retroviruses, Cell, **26**, 67-77.

Siegrist, W., Östreicher, M., Stutz, S., Girard, J., and Eberle, A.N. (1988) Radioreceptor assay for α-MSH using mouse B16 melanoma cells, J. Receptor Res., **8**, 323-343.

Simon, R.H., Engelhardt, J.F., Yiping, Y., Zepeda, M., Weber-Pendelton, S., Grossman, M., and Wilson, J.M. (1993) Adenovirus-mediated transfer of the CFTR gene to lung of non-human primates-toxicity study, Hum. Gene.Ther., **4**, 771-780.

Singer D.S., and Singer, M.F. (1978) Characterisation of complexes of superhelical and relaxed closed circular DNA with H1 and phosphorylated H1 histones, Biochemistry, **17**, 2087-2095.

Sipe, D.M., Jesurum, A., and Murphy, R.F. (1991) Absence of Na^+, K^+-ATPase regulation of endosomal acidification in K-562 erythroleukemia cells, J.Biol.Chem., **266**, 3469-3474.

Smith, T.A.G., Mehaffey, M.G., Kayada, D.B., Saunders, J.M., Yei, S., Trapnell, B.C., McClelland, A., and Kaleko, M. (1993) Adenovirus-mediated expression of therapeutic plasma levels of human factor IX in mice, Nat Genet., **5**, 397-402.

Smull, C.E., Mallette, and Ludwig, E.H. (1961) The use of basic proteins to increase the infectivity of enterovirus ribonucleic acid, Biochem.Biophys.Res.Comm., **5**, 247-249.

Smull, C.E., and Ludwig, E.H. (1962) Enhancement of the plaque-forming capacity of poliovirus ribonucleic acid with basic proteins, J.Bacteriol. **84**, 1035-1040.

Spooner, R.A., Deonarain, M.P., and Epenentos, A.A. (1995) DNA vaccination for cancer treatment, Gene Therapy, **2**, 173-180.

Soriano, P., Dijkstra, J., Legrand, A., Spanjer, H., Londos-Gagliardi, D., Roerdink, F., Scherphof, G. and Nicolau, C. (1983) Targeted and non-targeted liposomes for *in vivo* transfer to rat liver cells of a plasmid containing the proopioinsulin I gene, Proc.Nat.Acad.Sci.USA, **80**, 1068-1072.

Stankovics, J., Crane, A-M., Andrews, E., Wu, C.H., Wu, G.Y., and Ledley, F.D. (1994) Overexpression of human methylmalonyl Co A mutase in mice after *in vivo* gene transfer with asilaoglycoprotein/polylysine/DNA complexes, Hum. Gene Ther., **5**, 1095-1104.

Stoschek, C.E., and Carpenter, G. (1984) Down-regulation of the epidermal growth factor receptor: Direct demonstration of receptor degradation in human fibrobalsts, J.Cell.Biol., **91**, 1048-1053.

Stratford-Perricaudet, L.D., Makeh, I., Perricaudet, M. and Briand, P. (1992) Widespread long-term gene transfer to mouse muscle cells and heart, J.Clin.Invest., **90**, 626-630.

Sutter, G., and Moss, B. (1992) Nonreplicating vaccinia virus vector efficiently expresses recombinant genes, Proc.Natl.Acad.Sci.USA, **89**, 10847-10851.

Szoka, F.C. and Papahaadjopoulos, D. (1978) Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation, Proc.Nat.Acad.Sci.USA, **75**, 4194-4198.

Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications, Proc.Natl.Acad.Sci.USA, **76**, 4350-4354.

Toyoshima, K., and Vogt, P.K. (1969) Enhancement and inhibition of avian sarcoma viruses by polycations and polyanions, Virology, **38**, 414-426.

Tsuboi, M., Matsuo, K., and T'so, P.O.P. (1966) Interaction of poly-L-lysine and nucleic acids, J.Mol.Biol., **15**, 256-267.

Ulmer, J.B., Donelly, J.J., Parker, S.E., Rhodes, G.H., Felgner, P.L., Dwarki, V.J., Gromkowiski, S.H. *et al.* (1993) Hetrologous protection against influenzae virus by infection of DNA encoding a viral protein, Science, **259**, 1745-1749.

Van Assendelft, G.B., Hanscombe, O., Grosveld, F., and Greaves, G.R. (1989) The β-globin dominant control region activates homologous and hetrologous promoters in a tissue specific manner, Cell, **56**, 969-977.

Varga, J.M., and Asato, N. (1977) Melanotropin daunomycin conjugate shows receptor mediated cytotoxicity in cultured murine melanoma cells, Nature, **267**, 56-57.

Vile, R.G., and Hart, I.R. (1993) *In vitro* and *in vivo* targeting of gene expression to melanoma cells, Cancer Res., 53, 962-967.

Vile, R.G., and Russell, S.J. (1994) Gene transfer technologies for the gene therapy of cancer, Gene Therapy, **1**, 88-98.

Vogt, P.K. (1967) DEAE-dextran: enhancement of cellular transformation induced by avian sarcoma viruses, Virology, **33**, 175-177.

Wagner, E., Zenke, M., Cotten, M., Beug, H., and Birnstiel, M.L. (1990) Transferrin-polycation conjugates as carriers for DNA uptake into cells, Proc.Natl.Acad.Sci.USA, **87**, 3410-3414.

Wagner, E., Cotten, M., Foisner, R., and Birnstiel, M.L. (1991) Transferrin-polycation-DNA complexes: The effect of polycations on the structure of the complex and DNA delivery to cells, Proc.Natl.Acad.Sci.USA, **88**, 4255-4259.

Wagner, E., Zatloukal, K., Cotten, M., Kirlappos.H., Mechtler, K., Curiel, D.T. and Birnstiel, M.L. (1992a) Coupling of adenovirus to transferrin-polylysine-DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes, Proc.Natl.Acad.Sci.USA, **89**, 6099-6103.

Wagner, E., Plank, C., Zatloukal, K., Cotten, M., and Birnstiel, M.L. (1992b) Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: Toward a synthetic virus-like gene-transfer vehicle, Proc.Natl.Acad.Sci.USA, **89**, 7934-7938.

Wagner, E., Curiel, D., and Cotten, M. (1994a) Delivery of drugs, proteins and genes into cells using transferrin as a ligand for receptor mediated endocytosis, Adv.Drug Del.Rev., **14**, 113-135.

Wagner, E., Buschle, M., Cotten, M., Plank, C., Zauner, W., and Birnstiel, M.L. (1994b) Synthetic virus-like gene delivery systems, Proceed.Intern.Symp.Control.Rel.Bioact.Mater., **21**, 3-4.

Waring, M.J. (1965) Complex formation between ethidium bromide and nucleic acids, J.Mol.Biol., **13**, 269-282.

Washington, C. (1990) The stability of intravenous fat emulsions in total parenteral nutrition mixtures, Int.J.Pharm, **66**, 1-21.

Washington, C. (1992) Particle size analysis in pharmaceutics and other industries. Theory and practice, Ellis Horwood Ltd., Chichester, UK.

Weintraub, H., Cheng, P.F., and Conrad, K. (1986) Expression of transfected DNA depends on DNA topology, Cell, **46**, 115-122.

Welsh, M.J. (1990) Abnormal regulation of ion channels in cystic fibrosis epithelia, FASEB.J., **4**, 2718-2725.

Westphal, H., and Dulbecco, R. (1968) Viral DNA in polyoma- and SV 40-transformed cell lines, Proc.Nat.Acad.Sci.USA, **59**, 1158-1165.

Wicks, I.P., Howell, M.L., Hancock, T., Kohsaka, H., Olee, T., and Carson, D.A. (1995) Bacterial lipopolysacchride co-purifies with plasmid DNA: Implications for animal models and human gene therapy, Hum. Gene Ther., **6**, 317-323.

Wickstrom, E. (1986) Oligodeoxynucleotide stability in subcellular extracts and culture media, J.Biochem.Biophys.Methods, **13**, 97-102.

Wilson, J.M., Grossman, M., Cabrera, J.F., Wu, C.H. and Wu, G.Y. (1992) A novel mechanism for achieving transgene persistence *in vivo* after somatic gene transfer into hepatocytes, J.Biol.Chem., **267**, 11483-11489.

Wolff, J.A., Malone, R.W., Williams, P., Chong, W., Acsadi, G., Jani, G., and Felgner, P.L. (1990) Direct gene transfer into mouse muscle *in vivo*, Science, **247**, 1465-1468.

Wu, G.Y., and Wu, C.H. (1987) Receptor-mediated *in vitro* gene transformation by a soluble DNA carrier system, J.Biol.Chem., **262**, 4429-4432.

Wu, G.Y., and Wu, C.H. (1988a) Evidence for targeted gene delivery to Hep G2 hepatoma cells *in vitro*, Biochemistry, **27**, 887-892.

Wu, G.Y., and Wu, C.H. (1988b) Receptor-mediated gene delivery and expression *in vivo*, J.Biol.Chem., **263**, 14621-14624.

Yei, S., Mittereder, N., Tang, K., O'Sullivan, C., and Trapnell, B.C. (1994) Adenovirus-mediated gene transfer for cystic fibrosis: Quantitative evaluation of repeated *in vivo* vector administration to the lung, Gene Therapy, **1**, 192-200.

Yu, M., Poeschla, E., and Wong-Staal, F. (1994) Progress towards gene therapy for HIV infection, Gene Therapy, **1**, 13-26.

Zabner, J., Petersen, D.M., Pugh, A.P., Graham, S-M., Couture, L.A., Keyes, L.D., Lukason, M.J., St.George, J.A., Gregory, R.J., Smith, A.E., Welsh, M.J. (1994) Safety and efficacy of repetitive adenovirus-mediated cDNA administration to airway epithelia of primates and cotten rats, Nat.Genet., **6**, 75-83.

Zatloukal, K., Wagner, E., Cotten, M., Phillips, S., Plank, C., Steinlein, P., Curiel, D.T., and Birnstiel, M.L. (1992) Transferrinfection: A highly efficient way to express gene constructs in eukaryotic cells, Ann.N.Y.Acad.Sci., **660**, 136-153.

Zenke, M., Steinlein, P., Wagner., E., Cotten, M., Beug, H., and Birnstiel, M.L. (1990) Receptor-mediated endocytosis of transferrin-polycation conjugates: An efficient way to introduce DNA into hematopoietic cells, Proc.Natl.Acad.Sci.USA, **87**, 3655-3659.

Zhu, N., Liggitt, D., and Debs, R. (1993) Systemic gene expression after intravenous DNA delivery to adult mice, Science, **261**, 209-211.

Appendix 1: Media and Solutions

Where appropriate, media and solutions were sterilised by autoclaving for 15 minutes at 15 lbs/sq. inch (121°C) on a liquid cycle.

Buffer A: 20 mM Hepes/1M NaCl, pH 7.9.

Buffer B: 20 mM Hepes/2M NaCl, pH 7.9.

Buffer P1: 100 µg/ml RNase A, 50 mM Tris-HCl, 10 mM EDTA, pH 8.0.

Buffer P2: 200 mM NaOH, 1% $^{w}/_{v}$ SDS.

Buffer P3: 3.0 M Potassium acetate, pH 5.5.

Buffer QBT: 750 mM NaCl, 50 mM MOPS, 15% $^{v}/_{v}$ ethanol, 0.15% $^{v}/_{v}$ Triton X-100.

Buffer QC: 1.0 M NaCl, 50 mM MOPS, 15% $^{v}/_{v}$ ethanol.

Buffer QF: 1.25 M NaCl, 50 mM Tris-HCl, 15% $^{v}/_{v}$ ethanol.

DNA loading buffer: 50 mM EDTA, 0.2% SDS, 0.05% $^{w}/_{v}$ bromophenol blue, 50% glycerol.

Ethidium Bromide: 1 mg/ml in double distilled water.

Hepes Buffered Saline (HBS): 20 mM Hepes, 150 mM NaCl, pH 7.4.

Phosphate Buffered Saline (PBS): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3.

LB medium (Luria-Bertani medium): (per litre) 10 g Bacto tryptone (Difco), 5 g Bacto yeast extract (Difco) and 10 g NaCl, adjusted to pH 7.0 with NaOH. LB plates were made from 1 litre of LB plus 20 g agar (Difco).

RNAase A: 10 mg/ml pancreatic ribonuclease A (Sigma) in STE heated to 100°C for 5 minutes and allowed to cool to room temperature.

SM salts (2X): 20 mM Hepes, 275 mM NaCl, 5.4 mM KCl, 8.6 mM Na_2HPO_4, 2.8 mM KH_2PO_4, pH 7.3.

SOC medium: 2% Bacto-tryptone (Difco), 0.5% Bacto yeast extract (Difco), 10 mM NaCl, 2.5 mM KCl, 10mM $MgCl_2$, 1 mM $MgSO_4$, 20 mM glucose.

STE: 10 mM Tris-HCl, 15 mM NaCl, pH 7.5.

TAE (10X): (per litre) 48.4 g Tris-base, 11.4 ml glacial acetic acid, 20 ml 0.5 M EDTA, pH 8.0.

TBS: 0.9% $^{w}/_{v}$ NaCl in 10 mM Tris-HCl, pH 7.4.

TBS-Tween: 0.15% $^{w}/_{v}$ Tween 20 in TBS.

TE: 10mM Tris-HCl, 1 mM EDTA, pH 8.0.

Appendix 2: Molecular Weight Markers.

For agarose gel electrophoresis, markers consisted of *Hind* III-cut lambda DNA (Sigma) or *Eco*R1/*Hind* III-cut lambda DNA (Northumbria Biologicals). The digests consist of DNA fragments of the following sizes (Base pairs):

***Hind* III-cut λ DNA**		***Eco*R1/*Hind* III-cut λ DNA**	
1: 23130	5: 2322	1: 21226	8: 1584
2: 9416	6: 2027	2: 5148	9: 1375
3: 6557	7: 625	3: 4973	10: 947
4: 4268	8: 125	4: 4268	11: 831
		5: 3530	12: 564
		6: 2027	13: 125
		7: 1904	

For SDS-PAGE pre-stained markers (Bio-Rad) consisted of;

Protein	**Molecular Weight**
Phosphorylase B	106 kDa
Bovine serum albumin	80 kDa
Ovalbumin	49.5 kDa
Carbonic anhydrase	32.5 kDa
Soybean trypsin inhibitor	27.5 kDa
Lysozyme	18.5 kDa

Appendix 3: Calibration curves for quantitative assays.

A3.1. UV assay for streptavidin.

Serial dilutions were prepared from a 1 mg/ml solution of streptavidin and the following concentrations produced in HBS (pH 7.4): 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml. These gave a linear Beer-Lambert plot for absorbance at 280 nm (Fig. A3.1.) with the following linear regression analysis:

Slope = 1.29 $ml.mg^{-1}$ Intercept = -8×10^{-3} r = 0.9978

A3.2. Ninhydrin assay for primary amines.

A series of sample solutions were prepared by dilution of a 0.2 mg/ml stock solution of poly-L-lysine•HBr(219) with HBS. Samples were produced which contained poly-L-lysine equivalent to 0.5, 1.0, 1.5, 2.0, 2.5, and 3.5×10^{-7} moles of amine in 1.0 ml total volume. Analysis was performed as described in section 3.7.2. A linear Beer-Lambert plot for absorbance at 570 nm was determined (Fig. A3.2.) with the following linear regression analysis.

Slope = 1.1×10^{-1} μmol^{-1} Intercept = 2.1×10^{-3} r = 0.9982

A3.3. Spectrophotometric analysis of β-galactosidase activity in cell extracts.

A series of sample solutions were produced in 0.1 M sodium phosphate buffer from a commercial stock solution (1.06 units/ml). The samples, which contained β-galactosidase equivalent to 0, 2, 4, 6, 8, 10 and 12 milliunits of activity, were then assayed by the method described in section 4.2.2. A linear Beer-Lambert plot for absorbance at 420 nm was obtained (Fig. A3.3.) with the following linear regression analysis:

Slope = 5.6×10^{-2} $milliunits^{-1}$ Intercept = 5.1×10^{-3} r = 0.999

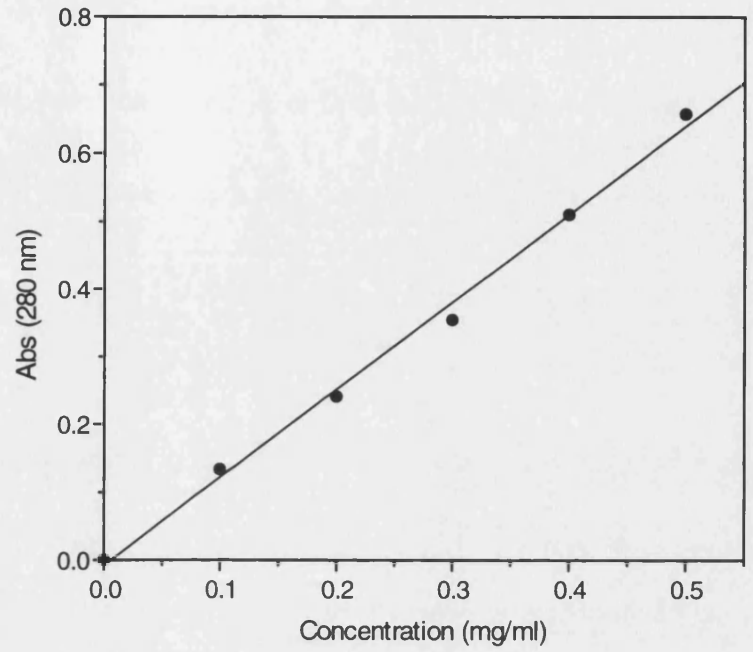


Figure. A3.1. Absorbance at 280nm (●) versus streptavidin concentration.

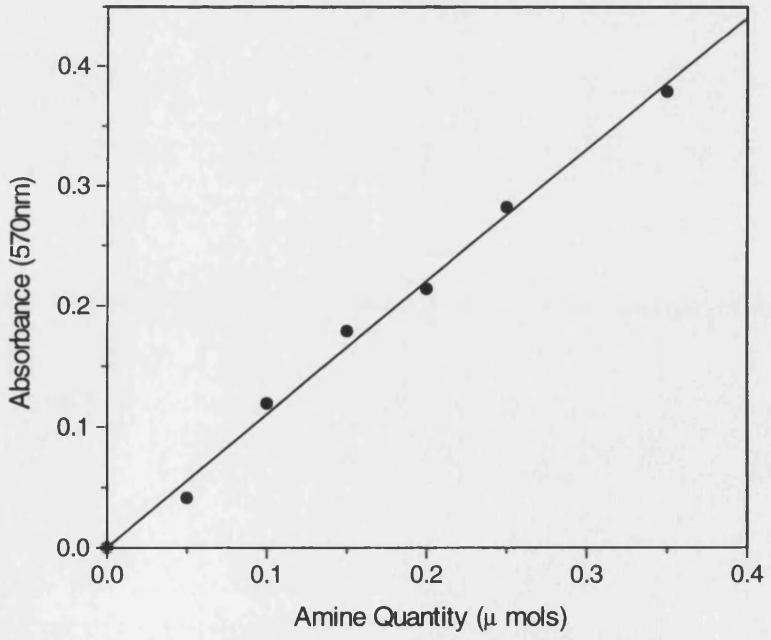


Figure. A3.2. Ninhydrin colour absorbance at 570nm (●) versus quantity of amine. Amine quantity was calculated using the DPn for poly-L-lysine(219).

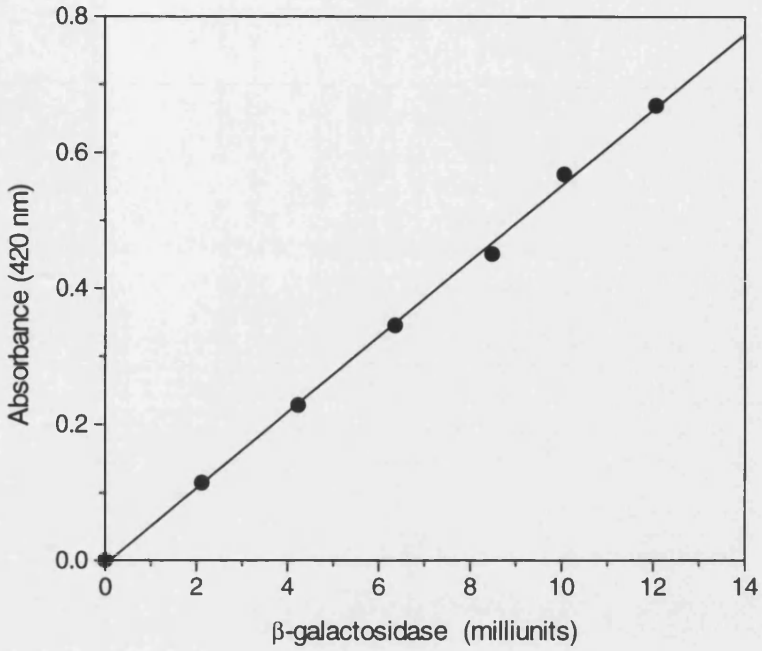


Figure A3.3. Absorbance at 420 nm versus β-galactosidase activity. Increasing quantities of β-galactosidase (milliunits activity) were incubated with assay reagent containing ONPG as described in section 4.2.2. Absorbance (●) was measured after 30 minutes.

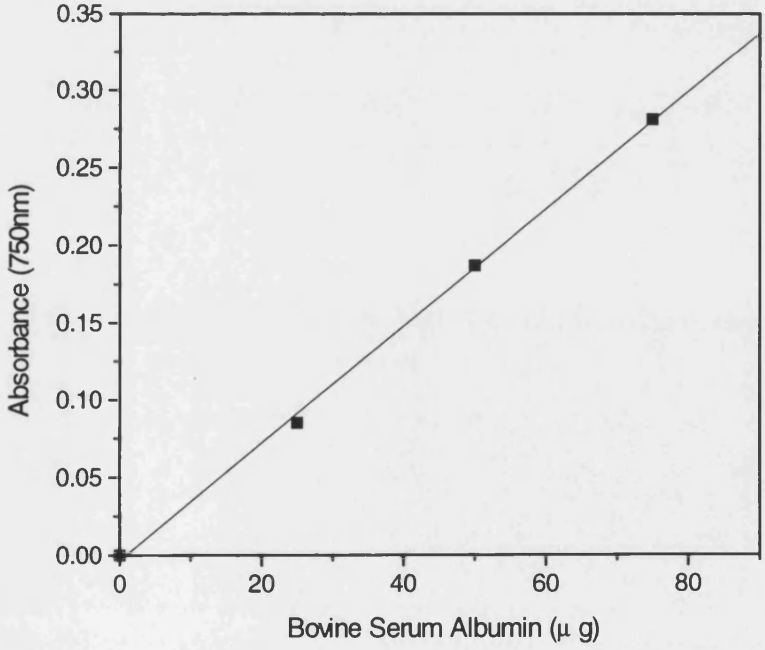


Figure A3.4. Standard curve for DC protein assay (Bio-Rad). Calibration was performed using bovine serum albumin.

A3.4. Assay for soluble protein content of cell extracts.

A standard curve for soluble protein content was determined using BSA (Sigma). From a 2 mg/ml stock solution of BSA 0.1 ml samples were prepared in 0.1M sodium phosphate buffer (pH 7.4) containing 0, 25, 50, and 75 μg of protein. Assayed by the method described in section 4.2.3. this gave a linear relationship between absorbance at 750 nm and protein content in the original sample (Fig. A3.4.). Above 100 μg protein a deviation from linearity was observed. The following linear regression analysis was obtained.

Slope = 3.8×10^{-3} μg^{-1} Intercept = -3.5×10^{-3} r = 0.999